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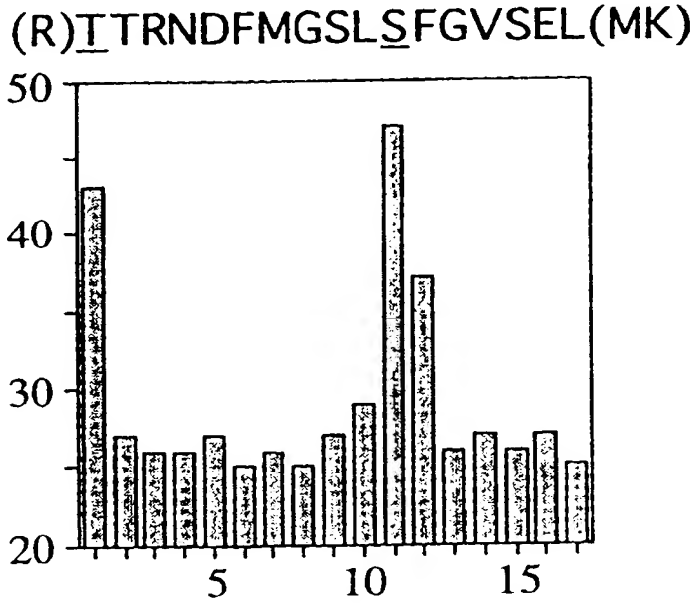

(57) Abstract

A method of determining whether a protein kinase C (PKC) has been activated, the method comprising determining whether or to what extent a phosphorylatable site on the said protein kinase C, which site is associated with activation of said protein kinase C, has been phosphorylated. A reagent which is capable of distinguishing between the presence or absence of a phosphate moiety at a phosphorylatable site in a protein kinase C, which site is associated with activation of said protein kinase C. Preferably, the reagent is an antibody.

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PROTEIN KINASE C

The present invention relates to protein kinase C (PKC), and in particular to a method of determining whether PKC has been activated and reagents for use in such a method.

The protein kinase C (PKC) gene family has been implicated *ex vivo* in the control of multiple cellular processes including cell division, differentiation and migration (see Nishizuka, 1989; Hug, 1993; Stabel, 1991). In general, PKC action in the context of such responses is thought to be elicited by the production of the second messenger diacylglycerol (DAG). Both the cPKC (α, β_1, β_2, γ) and nPKC (δ, ε, θ, η, μ) isotypes are responsive to DAG (reviewed [Dekker, 1994]). Evidence that activation of PKC follows DAG production is usually discerned through monitoring the amount of membrane-associated PKC either by activity or Western blotting. The latter provides a means of detecting the selective activation of particular isotypes. However, limitations of such analyses include the need to process native samples and the requirement for membrane-associated complexes to be stable to such processing. There is accumulating evidence that the stable association of PKC at membrane sites reflects not only direct-lipid interactions but also protein-protein contacts [Mochly-Rosen, 1995]; stability of the latter may have profound effects upon the classical "translocation" assay.

Previously we have shown that on stimulation of quiescent fibroblasts with PKC agonists, PKCα becomes hyperphosphorylated [Mitchell, 1989]. This suggested that the general phosphorylation state may prove a useful marker for activation, however ^{32}P-orthophosphate labelling has not been

the method of choice for analysis since undesirably large amounts of [^{32}P]-orthophosphate are required. Recent studies on the phosphorylation of cPKCs have shown that there are three phosphorylation sites that appear to be occupied to high stoichiometry and required for the optimal folding/activity of the protein [Tsutakawa, 1995; Keranen, 1995; Bornancin, 1996; Bornancin, 1997; Cazaubon, 1994]. I have now isolated a further phosphopeptide that shows a varied stoichiometry based upon recovery. The studies described here include the definition of the sites of phosphorylation present in this peptide and the development of antisera to one of these that proves to be an excellent marker for PKCα activation. Furthermore, similar activation markers for other PKCs, and reagents for detection of activated PKCs are described.

Many but not all of the PKC gene family bind and are activated by the phorbol ester class of tumour promoters (see Nishizuka, 1989; Stabel & Parker, 1991). These agents elicit a broad range of responses in biological systems including: tumour promotion, inflammation, irritation, cell division and cell differentiation (see Hug & Sarre, 1993). Such responses are considered to implicate PKC in many cellular controls. Coupled to the finding that many agonists induce an increase in the physiological second messenger DAG, PKC is expected to be involved in both physiological and pathological events. A key to defining involvement in these processes derives from establishing which specific PKC isotypes are actually activated under particular circumstances. Without such knowledge there is no basis upon which to determine (in advance) the usefulness of intervention.

A first aspect of the invention provides a method of determining whether a protein kinase C (PKC) has been activated, the method comprising determining whether or to what extent a phosphorylatable site on the said protein kinase C, which site is associated with activation of said protein kinase C, has been phosphorylated.

Protein kinase C is defined as a polypeptide encoding a C1A and/or B regulatory domain (defined in Hurley 1997) and a protein kinase catalytic domain (see Hanks 1995). Most PKC isotypes also encode a C2 or C2-like domain (see Ponting 1996), but these are not essential to the definition of a PKC.

Activated PKC may be defined as a PKC protein where through interaction with its allosteric activators or through interaction with certain proteins such as RACKS (receptors for activated C kinases, which are well known in the art, and which bind to PKC in the active conformation and may be used to trap PKC in the active conformation), its intramolecular inhibition is relieved permitting expression of efficient phosphotransferase activity.

Activated PKC includes a PKC bound or recently bound to its allosteric activator(s). Allosteric activators for PKC include diacylglycerol, phospholipids, some free fatty acids, Ca^{2+}, certain phorbol esters and detergents. Not all PKCs are activated by all allosteric activators. Pharmacological agents other than phorbol esters also activate (eg bryostatins, mezerin).

By "phosphorylatable site" we mean any site on a PKC which may be phosphorylated by the action of an enzyme and a suitable phosphate

donor, such as ATP. Thus, a "phosphorylatable site" may be a serine residue or a threonine residue within the PKC polypeptide which is phosphorylated to form phosphoserine and phosphothreonine, respectively. Not all threonine residues or serine residues are phosphorylatable sites and, as will become clear below, in the context of the invention the phosphorylatable site is one which is associated with activation of a particular PKC.

By the phosphorylatable site being a "site associated with activation of protein kinase C" we mean a site which is substantially phosphorylated when the PKC is substantially activated and which is not substantially phosphorylated when the PKC is not substantially activated. Typically, but not essentially, the site associated with activation of protein kinase C is an autophosphorylation site. An autophosphorylation site is a site in an enzyme which is a substrate for the same enzyme and becomes phosphorylated when the enzyme is active.

Autophosphorylation sites for PKCs can readily be determined, for example, by methods disclosed in the Examples in relation to PKC_{α}. Conveniently, the particular isoform of PKC for which it is desired to identify the autophosphorylation site is provided in a substantially pure form (at least substantially free of other protein kinases), for example by expressing a suitable PKC cDNA in a host cell and isolating the PKC enzyme. The substantially pure enzyme is then incubated with a suitable phosphate donor such as ATP in the absence of any other protein kinase. Conveniently, the donor (γ) phosphate of ATP is labelled with a radioactive phosphorus atom such as ^{32}P or ^{33}P. Incorporation of the donor phosphate into the PKC enzyme is monitored, and the identity of the site which has become phosphorylated may be determined using any

suitable method, such as by proteolytic or chemical cleavage of the PKC, followed by identification of the peptide containing the donor phosphate by FPLC analysis and radio detection, and sequencing of the identified peptide.

It will be appreciated that autophosphorylation may require the presence of suitable cofactors. For example, for the autophosphorylation of PKC_α it is preferred if a suitable amount of tetradecanoyl phorbol acetate and phosphatidyl serine is present. Similarly, it is preferred if a suitable amount of tetradecanoyl phorbol acetate and phosphatidyl serine is present for autophosphorylation of other cPKCs or nPKCs. For aPKCs, effectors are less well-defined but, typically, mixed brain phospholipids can activate to a limited extent and so it is preferred if they are present for the autophosphorylation of aPKC.

Suitable PKC enzyme preparations for carrying out autophosphorylation may be obtained using recombinant DNA technology as is well known in the art. Appropriate cDNAs encoding the PKC isozymes can be obtained by methods well known in the art by reference, for example, to the above mentioned GenBank accession numbers and records associated with the sequences in the data library.

Protein kinase C accession numbers:-

M94632	mouse PKCζ
D10495	human PKCδ
D90470	human PKCδ
D11091	mouse PKCθ
D28577	mouse PKCλ

J04532	rat PKCζ
D90242	mouse PKCή
J05703	mouse PKCή
M62980	mouse PKCή
L01087	human PKCθ
L07032	human PKCθ
L07860	human PKCδ
L07861	human PKCδ
L14283	human PKCζ
L28035	mouse PKCγ
L33881	human PKCι
M13706	rat PKC type II (β_I)
M13707	rat PKC type I (γ)
M13973	bovine PKCα
M13974	bovine PKCβ_1 (β_{II})
M13975	human PKCβ_1 (β_{II})
M13976	bovine PKCγ
M13977	human PKCγ
M18330	rat PKCδ
J03204	rat PKCδ
M18331	rat PKCε
J03204	rat PKCε
M20719	rat PKCε
M18332	rat PKCζ
J03204	rat PKCζ
M19007	rat PKCβ_1
M20014	rabbit PKCε
M22199	human PKCα
M25811	mouse PKCα

M55284	human PKC-L (η)
M69042	mouse PKCδ
J05335	mouse PKCδ
X04439	rat $PKC\beta_I$
X04440	rat $PKC\beta_{II}$
X04793	rabbit $PKC\beta_{II}$
X04795	rabbit $PKC\beta_I$
X04796	rabbit PKCα
X06318	human $PKC\beta_I$
M27545	human $PKC\beta_I$
X07109	human $PKC\beta_{II}$
X07286	rat PKCα
X07287	rat PKCγ
X52479	human PKCα
X52685	mouse PKCα
X51603	mouse PKCα
X53532	mouse $PKC\beta_{II}$
X59274	mouse $PKC\beta_I$
X60304	mouse PKCδ
X65293	human PKCε
X67129	mouse PKCγ
X68400	rat PKCη
X75756	human PKCμ
Z15108	human PKCζ
Z15114	human PKCγ
Z22521	human PKCδ

The DNA is then expressed in a suitable host to produce a PKC. Thus, the DNA encoding the PKC may be used in accordance with known techniques,

appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the PKC. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA encoding the PKC may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance.

Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors include a prokaryotic replicon, such as the ColE1 *ori*, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic, cell types. The vectors can also include an appropriate promoter such as a prokaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

Typical prokaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and p*Trc*99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

Useful insect cell vectors are the baculovirus vectors as can be obtained from Invitrogen, De Schelp 12, 9351 NV Leek, Netherlands.

A variety of methods have been developed to operably link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen

bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as is well known in the art, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3′-single-stranded termini with their 3′-5′-exonucleolytic activities, and fill in recessed 3′-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA encoding the PKC so that it may be readily introduced into an expression vector is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491.

In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

The host cell which is to express the PKC can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650. Insect cells, such as Sf9 and Hi5, are also useful eukaryotic host cells.

Transformation of appropriate cell hosts with a DNA construct encoding PKC is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110 and Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast*

Genetics, A Laboratory Manual, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* **275**, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming cells and is well known in the art for transforming yeast cells, bacterial cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* **2**, 637-646 incorporated herein by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5X PEB using 6250V per cm at 25μFD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct can be grown to produce the PKC. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985) *Biotech.* **3**, 208.

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological

methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Typically, phosphorylation of the phosphorylatable site associated with activation of said protein kinase C is not itself required for activation but it is a consequence of activation. Typically, compared to other sites in a PKC which may be phosphorylated, for example by the action of an upstream protein kinase, the site associated with activation exhibits a variable extent of phosphorylation which is a reflection of the extent of activation. Thus, as is disclosed in more detail in the Examples, PKC phosphopeptides which exhibit a varied stoichiometry based on recovery when isolated from their natural cells under different conditions may contain (as the site at which phosphorylation has occurred) a site associated with activation of said protein kinase C. Such sites may be identified using methods herein disclosed.

For PKC isotypes that display an activation-associated transphosphorylation at a site that is dephosphorylated on deactivation, these can also be employed as activation markers. Unlike autophosphorylation sites, the activation-associated transphosphorylation sites are conveniently defined empirically by identification following isolation of ^{32}P-phosphate labelled PKC from unstimulated and stimulated cells. It is also possible to identify such potential sites by, for example, random or precedent-directed mutagenesis or phospho-site antibody production. The usefulness of positively identified sites can be determined

in retrospect. For example, aPKCζ can be shown to be phosphorylated on a well conserved activation loop site at threonine 410. This phosphorylation is found to vary as a function of activation state (ie go up and down), and detection of phosphorylation (or the extent of phosphorylation) at this site, for example using antibodies specific for this site, is a determination of activation. Threonine 410 in a PKCζ is not an autophosphorylation site.

Because some phosphorylatable sites which serve as activation markers may be conserved between related protein kinases, specificity for any one unique gene product may be effected by use of a monospecific antibody that captures the protein kinase (eg ELISA, immunoprecipitation) while the activation specific antibody is employed as a second layer (eg ELISA) or, for example, in a subsequent western (immunoprecipitation).

Thus, in one preferred embodiment of the invention the method additionally comprises distinguishing the said PKC, whose activation state is to be determined by determining whether the said phosphorylatable site has been phosphorylated, from another PKC. It will be appreciated that the PKC whose activation state is to be determined can be distinguished from another PKC by contacting the sample with a compound which distinguishes the PKCs. Suitably, such a compound is a PKC-selective antibody. PKC-selective antibodies may be readily made by the person skilled in the art, for example by making use of the amino acid sequence differences between PKCs in order to raise antibodies. In particular, antibodies which are PKC-selective may be raised against PKC-specific peptides using methods well known in the art.

For example, the PKC-selective antibody may be used to separate the PKC whose activation is to be determined from other PKCs such as by immunoprecipitation, or it may be used to distinguish the PKCs.

The method of the invention may be used to determine whether a protein kinase C of any class has been activated. For example, it may be used for "a"-class (atypical) PKCs and for "c"-class ("classical)" PKCs and for "n"-class ("novel") PKCs.

The "a" class PKCs include PKC_ζ, PKC_ι and PKC_λ. PKC_ι and PKC_λ are human and mouse homologues. They are often referred to as $PKC_{\iota/\lambda}$.

The "c" class PKCs include PKC_α, $PKC_{\beta 1}$, $PKC_{\beta 2}$ and PKC_γ.

The "n" class PKCs include PKC_δ, PKC_ε, PKC_θ, PKC_η and PKC_μ.

The method of the present invention has significant advantages over the previously used "translocation" method for determining the activation of a PKC isoform. The basis of the "translocation" method was the observations that activation leads to a membrane-bound form of PKC and that activation is a membrane-binding step. The previous method, therefore, relied on determining the intracellular location of a particular PKC isoform. In contrast, with the present method, no cellular fractionation is required and the quality of the sample to be tested is not so important. In particular, the present method allows for historical or archival samples, such as frozen tissue samples or even samples stored as paraffin-fixed tissue sections, to be analysed as well as fresh tissue samples and cell lines.

It is particularly preferred if the method is used to detect activation of a human PKC but it may be used in relation to any PKC.

In a particularly preferred embodiment, the method comprises determining whether PKC_α or PKC_β, or $PKC_{\beta 2}$ or PKC_γ has been activated. I have found that threonine 250 (T250) of PKC_α is associated with activation of PKC_α and the phosphorylation status of T250 serves as an activation marker for PKC_α. Thus, the determination of the phosphorylation status of T250 of PKC_α provides an indication of the activation status of PKC_α. If T250 has been phosphorylated, PKC_α is activated; if T250 is not phosphorylated, PKC_α is not activated.

Priming sites that are probably not (*), and those that are likely to be (+), autophosphorylation sites for human PKCs:

human PKCδ	T505(*)	S662(*)	S643(+)
human PKCε	T566(*)	S729(*)	T710(+)
human PKCη	T512(*)	S674(*)	T655(+)
human PKCθ	T538(*)	S695(*)	S676(+)
human PKCζ	T410(*)	T560(+)	
human PKCι	T403(*)	T555(+)	

In some situations it is valuable to be able to determine not only the absolute amount of active PKC but also the proportion. Since there are many situations where changes in PKC concentration occur, absolute amounts of active PKC may provide insufficient information. For example, for samples from patients treated with PKC inhibitors it may be desirable to know the proportion of active PKC, to define how effective

drug treatment has been. Such measurements may be achieved, for example, by combining a simple two site ELISA (eg PKC capture using, for example, a PKC-selective antibody and activation state detection, for example using an antibody which detects phosphorylation of a phosphorylatable site associated with activation of that PKC) with a parallel reading to determine the quantity of PKC captured (using a PKC antibody to a second protein epitope). Phosphorylation of T410 in PKCζ, and phosphorylation of T403 in PKCι, are markers of the respective PKCs.

The amino acid sequence of human PKC_{α}, human $PKC_{\beta 1}$, human $PKC_{\beta 2}$ and rat PKC_{γ} is shown in Figure 10. A threonine residue equivalent to T250 of human PKC_{α} is conserved in human $PKC_{\beta 1}$, human $PKC_{\beta 2}$ and rat PKC_{γ}, and I have found that phosphorylation of the equivalent threonine residue (as shown in Figure 10) is also a marker for activation of human $PKC_{\beta 1}$, human $PKC_{\beta 2}$ and rat PKC_{γ}.

The determination of whether or to what extent said phosphorylatable site has been phosphorylated may be made using any suitable method, for example, by amino acid sequencing. However, it is preferred that the determination is made using a reagent which is capable of distinguishing between the presence or absence of a phosphate moiety at said phosphorylatable site.

Preferably, the reagent is an antibody or a suitable fragment or derivative thereof which is capable of distinguishing between the presence or absence of a phosphate at the phosphorylatable site.

By a "suitable fragment or derivative of an antibody", we include any molecule derived from an antibody or which has an antibody-like binding site, which can distinguish between the presence or absence of a phosphate in a PKC as said. Suitable fragments and derivatives include $F(ab')_2$ fragments, Fab fragments, ScFvs, domain antibodies (dAbs) and the like.

It is preferred that the antibody or suitable fragment or derivative thereof recognises and binds to the phosphorylated phosphorylatable site.

The antibody is, therefore, preferably one which binds to a specific phosphopeptide. It is preferred that the antibody does not bind to phosphoserine or phosphothreonine in the absence of further specific determinants in the polypeptide surrounding the specific phospho-residue.

Antibodies which cross-react with a PKC when phosphorylated at a specific site and which do not cross-react to any significant extent with the PKC when not phosphorylated at that specific site may be made using any suitable method. Antibodies may be raised against specifically phosphorylated PKC and screened for their ability to bind to said specific phosphorylated PKC and for their inability to bind to said specific non-phosphorylated PKC. However, the preferred method for making antibodies which are useful in the practice of the invention involves the use of phosphopeptides based on the primary amino acid sequence surrounding the phosphorylatable site in which the site is phosphorylated. For example, the peptide WDRT(P)TRND.amide, where T(P) denotes a phosphorylated threonine residue, is particularly useful for raising antibodies which react with human PKC_α phosphorylated at T250.

The antibodies may be monoclonal or polyclonal.

Monoclonals provide an easily renewable resource, polyclonals may require extensive characterisation (for each new batch). Polyclonals are usually of a different animal origin to monoclonals and this can be useful where two-site detection is required (obviating the need for monoclonals of distinct subclasses since secondary reagents such as antibodies can distinguish polyclonal antibodies from different animal sources (such as anti-sheep antibody antibodies, or anti-rabbit antibody antibodies)). It is preferable if there are a range of monoclonals of different IgG subclass, particularly when a two-stage system is used, since the positive binding of each subclass can be measured using a subclass-selective reagent.

With today's techniques in relation to monoclonal antibody technology, antibodies can be prepared to most antigens. The antigen-binding portion may be a part of an antibody (for example a Fab fragment) or a synthetic antibody fragment (for example a single chain Fv fragment [ScFv]). Suitable monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in *"Monoclonal Antibodies: A manual of techniques"*, H Zola (CRC Press, 1988) and in *"Monoclonal Hybridoma Antibodies: Techniques and Applications"*, J G R Hurrell (CRC Press, 1982).

Typically, the phosphopeptide may be prepared as glutaraldehyde coupled conjugates to keyhole limpet haemocyanin (KLH) or ovalbumin for immunisation; ELISA may be used to screen hybridomas; and western blotting may be used on activated or non-activated samples to confirm specificity of the monoclonal antibody.

Conveniently, the antibodies of the invention may be used in any suitable way to detect the presence of the specifically phosphorylated, and hence activated, PKC. For example, the antibodies may be used in Western blotting, or ELISA, or in *in situ* binding assays.

In-situ binding assays include those involving tissue sections, fixed cells, microinjected live cells with coincident detection of the protein using FRET (fluorescence resonance energy transfer) and related analyses. Additionally dot/slot blots, that is cell extract applied directly to a filter or appropriate support and then treated as a western, may be used. For this latter type of analysis, it is preferred if the reagents are of very high quality since no independent criteria are used, ie recognition by another antibody (ELISA) or size (western).

Methods for identifying the activation marker site for any given PKC are included in the invention. Conveniently, the most direct method for identifying marker sites in any given PKC is based upon autophosphorylation *in vitro*. Preferably, purified PKC is isolated and incubated in the presence of appropriate activators and Mg^{2+} -[^{32}P]ATP. Incorporation of ^{32}P into the PKC protein through autophosphorylation provides a marker for the newly phosphorylated site(s). The protein may be fragmented with a protease (trypsin is most useful, although others will also work) or through chemical cleavage (for example, using cyanogen bromide). The labelled protein fragment(s) are identified by monitoring the presence of ^{32}P following purification; this can be achieved by electrophoresis, thin layer chromatography, HPLC and other related column chromatographic methods or any other suitable method. The site may be identified by use of sequential Edman degradation using a commercially available automated device (as supplied by Applied

Biosystems) by one skilled in the art. The PTH-amino acids sequentially derived may be identified by HPLC; a proportion of the sample is employed to determine ^{32}P content. It may be desirable to use phosphoaminoacid analysis as an independent means of confirming the nature of the residue involved (i.e. serine, threonine or tyrosine). This can be effected through partial acid hydrolysis of the peptide and subsequent electrophoresis with phosphoaminoacid standards.

Phosphopeptide analysis can also be carried out by mass spectrometry. This can circumvent the need to purify and also the need to ^{32}P label, although 'before' and 'after' autophosphorylation analyses are needed to define the new site(s). For mass spectrometry it is possible to first enrich phosphopeptides by small scale metal chelate HPLC. The whole mixture of peptides is then subjected to mass spectroscopic analysis to determine mass and through fragmentation, sequence.

Additionally or alternatively a series of synthetic peptides that encompass each of the candidate serine residues may be made such that each peptide has a single serine, the others being altered to alanine. These may be tested as substrates for a protein kinase to determine which serine is phosphorylated. The phosphorylation site may also be identified using site directed mutagenesis.

Thus, a further aspect of the invention provides a method for identifying a marker associated with activation of a protein kinase C the method comprising comparing the phosphorylation state of a phosphorylatable site in activated said protein kinase C with that of the phosphophorylatable site in non-activated said protein kinase C and determining those

phosphorylatable sites whose phosphorylation state varies with the activation status of the protein kinase C.

A further aspect of the invention provides the use of a reagent which is capable of distinguishing between the presence or absence of a phosphate moiety at a phosphorylatable site in a protein kinase C, which site is associated with activation of said protein kinase C for determining whether a protein kinase C has been activated.

The reagent is preferably an antibody as disclosed above.

A still further aspect of the invention provides a reagent which is capable of distinguishing between the presence or absence of a phosphate moiety at a phosphorylatable site in a protein kinase C, which site is associated with activation of said protein kinase C.

Suitably, the reagent is an antibody or a suitable fragment or derivative thereof. The antibodies may be made as disclosed above. In particular, once the phosphorylated site which is a marker of activation has been identified, phosphopeptides which are used for raising antibodies may be readily made using methods well known in the art.

Peptides may be synthesised by the Fmoc-polyamide mode of solid-phase peptide synthesis as disclosed by Lu *et al* (1981) *J. Org. Chem.* **46**, 3433 and references therein. Temporary N-amino group protection is afforded by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. Repetitive cleavage of this highly base-labile protecting group is effected using 20% piperidine in N,N-dimethylformamide. Side-chain functionalities may be protected as their butyl ethers (in the case of serine, threonine and tyrosine), butyl esters

(in the case of glutamic acid and aspartic acid), butyloxycarbonyl derivative (in the case of lysine and histidine), trityl derivative (in the case of cysteine) and 4-methoxy-2,3,6-trimethylbenzenesulphonyl derivative (in the case of arginine). Where glutamine or asparagine are C-terminal residues, use is made of the 4,4'-dimethoxybenzhydryl group for protection of the side chain amido functionalities. The solid-phase support is based on a polydimethyl-acrylamide polymer constituted from the three monomers dimethylacrylamide (backbone-monomer), bisacryloylethylene diamine (cross linker) and acryloylsarcosine methyl ester (functionalising agent). The peptide-to-resin cleavable linked agent used is the acid-labile 4-hydroxymethyl-phenoxyacetic acid derivative. All amino acid derivatives are added as their preformed symmetrical anhydride derivatives with the exception of asparagine and glutamine, which are added using a reversed N,N-dicyclohexyl-carbodiimide/1-hydroxybenzotriazole mediated coupling procedure. All coupling and deprotection reactions are monitored using ninhydrin, trinitrobenzene sulphonic acid or isotin test procedures. Upon completion of synthesis, peptides are cleaved from the resin support with concomitant removal of side-chain protecting groups by treatment with 95% trifluoroacetic acid containing a 50% scavenger mix. Scavengers commonly used are ethanedithiol, phenol, anisole and water, the exact choice depending on the constituent amino acids of the peptide being synthesised. Trifluoroacetic acid is removed by evaporation *in vacuo*, with subsequent trituration with diethyl ether affording the crude peptide. Any scavengers present are removed by a simple extraction procedure which on lyophilisation of the aqueous phase affords the crude peptide free of scavengers. Reagents for peptide synthesis are generally available from Calbiochem-Novabiochem (UK) Ltd, Nottingham NG7 2QJ, UK. Purification may be effected by any one, or a combination of, techniques such as size exclusion chromatography, ion-exchange chromatography and

(principally) reverse-phase high performance liquid chromatography. Analysis of peptides may be carried out using thin layer chromatography, reverse-phase high performance liquid chromatography, amino-acid analysis after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis.

The phosphate esters are alkali labile and to some extent acid labile. They do not survive complete acid (6N HCl) hydrolysis of peptides, but can be identified on partial hydrolysis (110°C, 1h 15mins). It is routine to analyse synthetic peptides using matrix assisted laser description mass spectrometry. This is a time of flight (ToF) device that gives precise mass measurements. For synthetic peptides this provides confirmation of predicted sequence (can not distinguish leucine and isoleucine) and assessment of purity is also obtained.

The peptides, when used as immunogens, may be present as single copies or as multiples, for example tandem repeats. Such tandem or multiple repeats may be sufficiently antigenic themselves to obviate the use of a carrier. It may be advantageous for the peptide to be formed as a loop, with the N-terminal and C-terminal ends joined together, or to add one or more Cys residues to an end to increase antigenicity and/or to allow disulphide bonds to be formed. If the peptide is covalently linked to a carrier, preferably a polypeptide, then the arrangement is preferably such that the peptide of the invention forms a loop.

According to current immunological theories, a carrier function should be present in any immunogenic formulation in order to stimulate, or enhance stimulation of, the immune system. It is thought that the best carriers embody (or, together with the antigen, create) a T-cell epitope. The

peptides may be associated, for example by cross-linking, with a separate carrier, such as serum albumins, myoglobins, bacterial toxoids and keyhole limpet haemocyanin. More recently developed carriers which induce T-cell help in the immune response include the hepatitis-B core antigen (also called the nucleocapsid protein), presumed T-cell epitopes such as Thr-Ala-Ser-Gly-Val-Ala-Glu-Thr-Thr-Asn-Cys, beta-galactosidase and the 163-171 peptide of interleukin-1. The latter compound may variously be regarded as a carrier or as an adjuvant or as both. Alternatively, several copies of the same or if appropriate different peptides may be cross-linked to one another; in this situation there is no separate carrier as such, but a carrier function may be provided by such cross-linking. Suitable cross-linking agents include those listed as such in the Sigma and Pierce catalogues, for example glutaraldehyde, carbodiimide and succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, the latter agent exploiting the -SH group on any cysteine residue (if present).

Preferably, the antibodies or other reagents of the invention are detectably labelled. By "detectably labelled" we mean that the antibody or other reagent is labelled in such a manner that it can be readily detected; for example, the labelling may be direct such as radiolabelling, or fluorescent or coloured labelling; or the labelling may be indirect such as labelling with an enzyme-linked system in which the enzyme converts an uncoloured substrate into a coloured product.

A further aspect of the invention provides a kit of parts comprising a reagent which is capable of distinguishing between the presence or absence of a phosphate moiety at a phosphorylatable site in a protein kinase C, which site is associated with activation of said protein kinase C and, as

controls, a sample comprising activated said protein kinase C and a sample comprising non-activated said protein kinase C.

Conveniently, the reagent of the kit is an antibody as herein described. Preferably, the controls are provided by cell extracts comprising said protein kinase C wherein said cells have been stimulated (to produce activated said PKC) or unstimulated (to produce non-activated said PKC).

It will be appreciated that it is preferred that the kit further comprises a reagent which distinguishes the said PKC whose activation state is to be determined from another PKC. For example, it is herein disclosed that at least some antibodies which are useful in distinguishing between the presence or absence of a phosphate moiety at a phosphorylatable site in a PKC, which site is associated with activation of said protein kinase C, may also detect the presence or absence of a phosphate moiety at a phosphorylatable site in a different, but related, PKC. In this case, it is preferred to use an antibody, unrelated to the antibody which detects the presence or absence of phosphorylation at the phosphorylatable activation marker site, in order to distinguish the different but related PKCs.

Typically, a kit might contain: an activation marker antibody; an antibody to the specific protein kinase C; a protein kinase C transfected cell extract from (i) unstimulated and (ii) stimulated cells as a control; and, optionally, a second layer detection conjugate (optional). If both antisera are from the same species, it is desirable to mark the detecting, activation marker antibody, with for example biotin. This would permit the second layer detection to be some suitable streptavidin-X complex, where X is any convenient detection system such as alkaline phosphatase or horse radish peroxidase.

Conveniently, the kit further comprises directions on use.

It will be appreciated that the methods of the invention may be used on fresh material, such as cultured cells or tissue samples (once suitably prepared) or it may be used on archival or historic material (once suitably prepared). The methods and reagents of the invention may be used whenever it is desirable to determine the activation status of a protein kinase C. This may be useful in the research laboratory for determining the effects of reagents or other conditions on PKC. It may also be useful in clinical and other situations.

A still further aspect of the invention provides a peptide comprising a phosphorylatable site in a protein kinase C which site is associated with activation of said protein kinase C, or a peptide in which said phosphorylatable site is phosphorylated.

Such peptides are useful for raising antibodies for use in the methods and kits of the invention. Conveniently, the peptide is an immunogenic portion of the PKC (whether phosphorylated at the phosphorylatable site or not) and typically has between 5 and 50 amino acids, preferably between 6 and 40, more preferably between 7 and 30 and still more preferably between 8 and 20 amino acids. It is particularly preferred if the peptide comprises the phosphorylatable site which is found at T250 in bovine PKC_{α} or site equivalent thereto as herein disclosed. A preferred such peptide is the phosphorylated peptide WDRT(P)TRND.

The peptides are suitably included in an immunogenic formulation for raising antibodies. Preferably, the immunogenic formulation comprises the

antibody and a suitable carrier or adjuvant and, desirably, is sterile and pyrogen-free.

It is particularly desirable in sample preparation that substantially no proteolysis, substantially no dephosphorylation and substantially no phosphorylation take place. Preferably, this may be achieved by extraction into buffers with appropriate preservatives (eg phenylmethylsulfonyl fluoride, aprotinin, leupeptin, benzamidine, all of which are protease inhibitors; microcystin, okadaic acid and sodium fluoride + EDTA, all of which are phosphatase inhibitors; EDTA to chelate Mg^{2+} and so inhibit kinases). For simplicity it is particularly preferred to denature samples directly with 1% SDS. For subsequent analysis these may be diluted 10-fold with 1% Triton X-100 and the resulting detergent mixed micelles (1%Triton X-100, 0.1% SDS) do not interfere with PKC capture (ELISA) or direct immunoprecipitation.

In particular, it is useful to measure PKC activation by the methods of the invention in order to understand when PKC is triggered physiologically; or define which PKC is triggered physiologically; or determine where PKC is becoming activated (subcellular); or to follow efficacy of drug treatments directed at or through PKC; or to assess PKC action in (human) diseases and normal tissues.

The invention will now be described in more detail with respect to the following Examples and Figures wherein:

Figure 1 shows the identification of two PKCα phosphorylation sites. The histogram shows the ^{32}P radioactivity (counts per minute, cpm) released

from a ^{32}P-labelled PKCα tryptic peptide subjected to sequential Edman degradation. The counts are released at cycles 1 and 11. Based upon the specificity of trypsin (cleavage C-terminal to arginine, R and lysine, K) it can be predicted that the peptide is the one shown. The underscored amino acids are threonine (T) 250 and serine (S) 260.

Figure 2 shows that PKCα threonine 250 is phosphorylated in response to activation. COS cells transfected with PKCα were stimulated for 0, or 30 minutes with TPA in the absence (-) or presence (+) of a PKC inhibitor (10 μM bisindolylmaleimide I). The phosphorylation of PKCα is detected by Western analysis of whole cell extracts with a polyclonal antiserum specific for the phosphorylated T250 site. The lower panel shows that the amount of PKCα protein does not vary as evidenced by immunoreaction with a PKCα antiserum (MC5). Recognition of PKCα by the antiserum MC5 is independent of the phosphorylation state of PKCα.

Figure 3 shows that threonine 250 is a PKCα autophosphorylation site. PKCα protein was purified from transfected COS cells and incubated for the times indicated with Mg-ATP in the presence of TPA and phosphatidylserine (allosteric activators). Reactions were terminated by denaturation and subjected to Western analysis. The upper panel shows immunoreactivity with the phosphorylated threonine 250 (T(P)250) specific antiserum. The lower panel demonstrates that the amount of PKCα does not alter.

Figure 4 shows PKCα autophosphorylation on threonine 250 in COS cells. (A) shows that TPA induces a time-dependent increase in the phosphorylation of COS cell expressed PKCα. The upper panel is a

Western of whole cell extracts from samples treated with TPA for the times indicated. The lower panel shows that the amount of PKCα does not vary during the first 30 minutes. By 60 minutes there is some downregulation, consistent with both a loss of PKCα immunoreactivity and of T(P)250 immunoreactivity. (B) shows that the protein identified in PKCα expressing COS cell extracts is PKCα. The PKCα protein was immunoprecipitated from cell extracts (using a PKCα antibody, MC5) and then subjected to Western analysis. The immunoreactivity with the T(P)250 antiserum is shown. As for Figure 4A, there is an increase in phosphorylation during the first 30 minutes. This is followed by a decline, reflecting downregulation of the protein.

Figure 5 shows that TPA induces endogenous PKCα threonine 250 phosphorylation in Swiss 3T3 cells. Quiescent Swiss 3T3 cells were treated with TPA (400 nM) for the times indicated and whole cell extracts subjected to Western blotting with the T(P)250-specific antiserum. The upper panel shows T(P)250 serum immunoreactivity; the specific (phosphopeptide competable) PKCα band is indicated by the arrow (the unmarked faster migrating band is variably observed and is not competed). The lower panel shows that over this time course there is no change in total PKCα content of the cells.

Figure 6 shows that threonine 250 phosphorylation is reversible. Swiss 3T3 cells were treated with PDBu for 20 minutes and then washed with phorbol dibutyrate (PDBu)-free medium (at 4°C) and incubated further at 37°C for the times indicated. Extracts were prepared and subjected to Western blotting with the T(P)250-antiserum. Immunoreactivity was quantified following scanning of autoradiographs. There is a lag in

immunoreactivity loss before an exponential decline; note the immunoreactivity is shown as a log scale.

Figure 7 shows that mitogens stimulate PKCα autophosphorylation in Swiss 3T3 cells. Quiescent Swiss 3T3 cells were treated for the times indicated with platelet derived growth factor (PDGF), bombesin or vasopressin as indicated. Cell extracts were prepared and subjected to Western blotting. The specific T(P)250 positive PKCα band is indicated by the arrows for each panel.

Figure 8 shows that PKCα phosphorylation on T250 is blocked by a PKC inhibitor. Quiescent Swiss 3T3 cells were stimulated for 10 minutes in the presence (+) or absence (-) of 10μM bisindolylmaleimide I (BIM). The agonists are: bombesin (Bom.), vasopressin (Vasop.) and PDGF. The arrow indicates the immunoreactive PKCα protein.

Figure 9 shows that insulin stimulates PKCα autophosphorylation in Swiss 3T3 cells. Quiescent Swiss 3T3 cells were treated with insulin (10^{-7}M) for the times indicated. Whole cell extracts were prepared and subjected to Western blotting with the T(P)250 serum or PKCα antibody as indicated.

Figure 10 is an alignment of the amino acid sequences of human PKC_{α}, $_{\beta 2}$ and $_{\beta 1}$ and rat PKC_{γ}. A "*" indicates perfect correspondence in amino acid; a ":" indicates a conservative amino acid substitution; a "." indicates related amino acid substitutions. Phosphorylation sites in PKC_{α} are marked with an arrow (T250; T497; T638; and S657).

Figure 11. Activated PKCα is selectively recognised by the T(P)250 antiserum *in situ*. Swiss 3T3 cells were processed as described in the Methods Section of Example 1. Control cells were untreated (con) or treated for 10 minutes with TPA (400 nM) as indicated. Following treatment cells were fixed and stained for PKCα (monoclonal 9E10) or for T(P)250 (PPA245 polyclonal). In addition to activated PKCα, the latter revealed a non-PKCα nuclear reaction in both uninjected (arrowhead) and injected cells (see text). Similar results were obtained employing an affinity purified PPA182, T(P)250 antiserum.

Figure 12 shows a section of a breast tumour showing T(P)250 staining quite intensely the tumour and much less so the surrounding tissue.

Figure 13 shows that a PKCζ is poorly phosphorylated at threonine 410 under normal cell culture conditions, but this can be greatly enhanced by stimulation of cells with the broad specificity agonist okadaic acid.

Example 1: Mapping a novel autophosphorylation site on protein kinase C α identifies an activation marker.

Investigation into the phosphorylation state of PKCα *in vivo* has led to the identification of two novel sites, threonine 250 (T250) and serine 260 (S260). Antisera specific for the occupied T(P)250 site were developed and used to demonstrate that this residue becomes phosphorylated on activation of PKCα by tetradecanoyl phorbol acetate (TPA) in transiently transfected COS-7 cells. This increased phosphorylation is inhibited by the PKC inhibitor, bisindolylmaleimide, consistent with an autophosphorylation process. Formal proof that T250 is an

autophosphorylation site was obtained with purified PKCα. The T(P)250-specific antisera have been employed to monitor PKCα activation in quiescent Swiss 3T3 cells. TPA, PDGF, bombesin and vasopressin were all found to induce T250 phosphorylation in a time- and dose-dependent manner, consistent with known targets and coupling mechanisms. Further, it is shown that insulin also induces T250 phosphorylation, demonstrating control of PKCα by this agonist. These studies reveal a novel activation-dependent phosphorylation site on PKCα that can serve as an activation marker; this has profound implications for analysis of compartmental activation and also archived pathological samples.

Experimental Procedures

Cell culture and transfection

COS-7 cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% foetal calf serum at 37°C and in a 10% CO_2 atmosphere. Cells were transfected by electroporation as described previously [Bornancin, 1996]. Transfected cells were stimulated as indicated in the text and figure legends, 48 hours after transfection. Where indicated bisindolylmaleimide I (Calbiochem) was added 20 minutes prior to agonist treatment. Following stimulation, cells were then harvested directly into Laemmli sample buffer [Laemmli, 1970]. For the purification of COS-7 cell expressed PKCα, the His-tagged recombinant protein was processed as described previously [Bornancin, 1996].

Swiss 3T3 cells were maintained at 37°C in a 10% CO_2 incubator in medium containing 10% foetal calf serum. Three days following seeding, cells were switched to DMEM containing 31% Weymouth's medium and

6% foetal calf serum; eight days after seeding cells were judged to be quiescent (see [Olivier, 1992]). Subsequent treatments are as indicated in the text and figure legends. Control or treated cells were harvested directly into Laemmli sample buffer for Western analysis.

Phosphorylation site mapping

Transfected (His-tagged PKCα) COS-7 cells were labelled with ^{32}P-orthophosphate for 6 hours at 2mCi/ml DMEM (phosphate-free) in the presence of 10% foetal calf serum. Cells were then rinsed twice with Tris-buffered saline (4°C) and harvested for PKCα purification as described previously [Bornancin, 1996]. Purified PKCα was further fractionated by SDS-PAGE and the labelled protein identified by autoradiography. Tryptic and VS protease-derived peptides were subsequently separated by HPLC and analysed by Edman degradation and/or 2-D peptide mapping (essentially as described previously [Oehrlein, 1996]). In some 2-D peptide maps the first dimension was run in formic acid at pH 1.9. Following Edman degradation, the PTH-amino acids released were collected and analysed for [^{32}P]-orthophosphate content; insufficient material was available for PTH-amino acid identification.

Antisera and Western blotting

To obtain antibodies to the two putative phosphorylation sites, the following synthetic phosphopeptides were synthesised in the Peptide Synthesis Laboratory (ICRF, London): GSLS(P)FGVSamide, WDRT(P)TRNDamide, where the S(P) and T(P) denote the phosphorylated residues S260 and T250 respectively (see text). Phosphopeptides were coupled to keyhole limpet haemocyanin using

glutaraldehyde and the conjugate employed to immunise rabbits. The sera obtained were employed for the studies described here.

Western blotting of immobilised proteins was carried out as described previously [Hansra, 1996] except that Tris-buffered saline pH 7.5 was used in place of phosphate-buffered saline. Antibodies were employed at 1/2000 for 1 hr at room temperature or overnight at 4°C. Immunoreaction was detected using ECL (Amersham) according to recommended procedures.

Microinjection of Swiss 3T3 cells and dual colour immunofluorescence/confocal microscopy

Swiss 3T3 fibroblasts ($8x10^6$) in Eagle's medium containing 10% fetal calf serum were plated on 10 cm petri dishes. After 3 days of culture, a third of the culture medium was replaced with serum-free Weymouth's Medium and the cells were cultured to quiescence for a further 5 days.

Subconfluent, quiescent cells were prepared as described elsewhere [Olson, 1996]. Cells were then trypsinized from culture dishes and reattached onto coverslips in serum-free media containing Type 1-S soybean trypsin inhibitor (Sigma) at 0.5 mg/ml. At 1h following reattachment, cells were microinjected with an expression plasmid pcDNA3 (Invitrogen) containing a myc-tagged full-length human PKCα cDNA construct and cultured for a further 2h at 37°C before stimulation with TPA (400 nM) for 10 min. Double-label immunofluorescence staining with the anti-Myc mAb 9E10 [Evan, 1985] and T(P)250 antiserum (PPA245) was performed as described [Kiley, 1997] except for the following modifications. Cells were permeabilised with 0.2% Triton-

X-100/PBS following fixation in 4% paraformaldehyde. Both primary antibodies were diluted 1:200 in 10 mM Tris-buffered saline containing 1% BSA. The secondary conjugates used were Cy2-conjugated donkey anti-mouse IgG (1:200) and Cy3-conjugated donkey anti-rabbit IgG (1:400) (Jackson ImmunoResearch Laboratories, West Grove, PA). Confocal images were acquired on a confocal laser scanning microscope (model SM410, Carl Zeiss Inc) equipped with a triple line Ar/Kr laser with a 100x1.4 NA Planapochromat oil immersion objective. Each image represents a 2-dimensional projection of sections in the Z-series, taken across the depth of the cell at 0.5 μm intervals.

See Figure 11.

Other Methods

Autophosphorylation of purified PKCα was carried out in the presence of tetradecanoyl phorbol acetate (2μM), phosphatidylserine (1.25mg/ml) in 1% (v/v) Triton X-100, 50 mM Hepes pH7.5, 12.5 mM Mg^{2+}, and 100 μM ATP. Reactions were initiated with ATP and terminated with Laemmli sample buffer at the times indicated.

Protein concentration was determined by the method of Bradford [Bradford, 1976] using bovine serum albumin (Sigma) as a standard.

Results

Orthophosphate labelling of PKCα transiently transfected COS-7 cells has revealed a number of *in vivo* phosphorylation sites including the well characterised threonine 497 (T497), threonine 638 (T638) and serine 657

(S657). In addition to these, one labelled tryptic peptide identified was found to be present to a variable degree. This peptide was HPLC purified and subjected to Edman degradation yielding [^{32}P-] phosphate release at cycles 1 and 11 (Figure 1). Inspection of the PKCα sequence indicated that a partially cleaved peptide from threonine 250 to lysine 268 most probably accounted for the peptide.

In order to establish the occupation and behaviour of these putative phosphorylation sites, antibodies were raised to phospho-peptides based upon the primary sequence. Studies with the S(P)260 specific antisera have demonstrated the presence of phosphate at this site, however to date no clear changes have been observed (data not shown). By contrast the T(P)250 specific antisera react with PKCα following stimulation of transfected COS-7 cells with TPA (Figure 2, upper panel). At this exposure, no immunoreaction is observed prior to stimulation and furthermore, inclusion of the PKC inhibitor BIM, blocks accumulation of the immunoreactive protein, indicative of an autophosphorylation. Reprobing the blot with a PKCα protein-directed monoclonal antibody (MC5) demonstrates that there is no acute change in the amount of protein present (Figure 2, lower panel).

To establish whether PKCα can autophosphorylate at this T250 site, COS-7 cell expressed His-tagged protein was purified as described previously [Bornancin, 1996]. Following incubation under phosphorylating conditions, the protein becomes phosphorylated in a time dependent manner at the T250 site, as judged by increased immunoreaction with the T(P)250-specific antiserum (Figure 3). Combined with the effect of BIM in TPA-induced phosphorylation *in vivo*, this indicates that

phosphorylation at the T250 site occurs by an autophosphorylation mechanism.

In transfected COS-7 cells, the induction of T250 phosphorylation of PKCα is time-dependent with a maximum response observed at 30 minutes (Figure 4A and 4B). This parallels the accumulation of a slower migrating form of the protein evidenced by immunodetection of the polypeptide. By 60 minutes the protein has become partially downregulated coincident with a parallel loss of immunoreaction with the T(P)250 antisera. To obtain direct evidence that the protein detected by this site-specific antiserum is PKCα, extracts were subjected to immunoprecipitation with the MC5 monoclonal antibody prior to Western analysis. As shown in Figure 4B, the site-specific antiserum detects the time-dependent phosphorylation of PKCα.

Immunodetection is reduced at longer times due to the downregulation of the protein (see Figure 4A).

In order to assess the use of the T(P)250-specific serum in monitoring activation of endogenous PKCα in cultured cells, quiescent Swiss 3T3 cells were employed. Stimulation of these cultures with TPA revealed an acute accumulation of immunoreactive protein (Figure 5, upper panel). In these cells, maximum phosphorylation was obtained within 5 minutes, with no change in recovered PKCα protein as determined by immunoreaction with MC5 (lower panel). This demonstrates that PKCα autophosphorylation can be employed as a marker for activation. The usefulness of this approach to monitoring PKCα activation may however be limited by the turnover of phosphate in this site - if turnover is slow or

absent, then immunoreaction might indicate an "historical" event. To address this, Swiss 3T3 cells were treated with the more hydrophilic phorbol ester phorbol dibutyrate (PDBu) and then washed free of agonist. As shown in Figure 6, removal of PDBu led to a lag period followed by a time-dependent loss of T(P)250 reactivity. The extent of the lag prior to dephosphorylation was variable indicative of slow re-equilibration of cellular PDBu. Once initiated however dephosphorylation occurs with a half-life of ~5 minutes.

Previous studies relying upon translocation (see [Farrar, 1985]) have demonstrated the activation of various PKC isotypes in response to agonists inducing DAG production, principally through activation of phosphoinositide-specific phospholipase C (PI-PLC). The PDGF receptor is coupled to PI-PLCγ, (amongst other activities) and has been shown to induce phosphoinositide (PI) hydrolysis in Swiss 3T3 cells (for example [Sturani, 1986]. Consistent with this, PDGF treatment of quiescent Swiss 3T3 cells was found to cause autophosphorylation of PKCα at the T250 site (Figure 7 upper panel). This effect was optimal at 1 ng/ml PDGF (not shown). Phosphorylation was time-dependent being maximal by 5 minutes, sustained for a further 5 minutes with dephosphorylation occurring after 10 minutes. This method of assessment of PKCα activation can thus be employed to monitor the action of physiological agonists. To further establish this point, other well studied agonists in Swiss 3T3 cells were also analysed. Both vasopressin and bombesin were found to induce time dependent increases in PKCα T250 phosphorylation (Figure 7 lower panels). To corroborate the autophosphorylation nature of the PKCα response, the effect of the PKC inhibitor BIM was determined. For all three agonists, BIM was found to block the T250 phosphorylation

of PKCα (Figure 8). These results indicate that PKCα activation by a range of agonists can be monitored through its autophosphorylation at the T250 site.

There has been much debate concerning the control of PKC by insulin (see [Blackshear, 1991] and references therein). To address this issue directly, we have monitored the effect of insulin on T250 phosphorylation in quiescent Swiss 3T3 cells. Stimulation by insulin was found to induce a time-dependent increase in PKCα phosphorylation as indicated by T(P)250 immunoreactivity (Figure 9).

Discussion

The study here, provides direct evidence for the phosphorylation of PKCα residue T250 in COS-7 cells and identifies this as an autophosphorylation site. Antisera specific to the occupied site (T(P)250) are characterised and shown to provide a means of following PKCα activation directly. The definition of the T250 site as an autophosphorylation site is based upon direct analysis of purified PKCα. *In vitro*, the COS-7 cell expressed and purified protein phosphorylates the T250 site on incubation with Mg-ATP and lipid activators. This is evidenced by increased immunoreactivity with the T(P)250-specific antisera and parallels an increase in ^{32}P-orthophosphate incorporation into the protein. It should be noted that the T250 site may not be the sole site autophosphorylated *in vitro*, but that its analysis here establishes the principle of an activation marker. Consistent with the *in vitro* data, it is found that the agonist induced phosphorylation of the T250 site in PKCα is inhibited by the PKC inhibitor BIM. Thus, although activation through ligand binding may induce a conformation (or

localisation) susceptible to T250 phosphorylation by an heterologous activity *in vivo*, the inhibition by BIM is consistent with autophosphorylation.

The studies on the T250 site have relied upon the use of antisera that recognise this phosphorylated epitope selectively. This is clearly demonstrated by comparing immunoreactivity of the serum in control and TPA-treated COS cells with that for PKCα protein (for example Figure 2). The fact that in these cells, PKCα is already phosphorylated at T497 and T638 (as well as S657) [Bornancin, 1996; Bornancin, 1997] demonstrates that this antiserum does not detect phosphothreonine in a non-specific manner but requires local sequence determinants.

The establishment of the T250 site as an autophosphorylation site and the development of selective antisera have provided a rationale for assessing activation *in vivo*. The efficacy of this approach is dependent upon the turnover of phosphate at this site and the detectability of endogenous phosphorylated PKCα (as opposed to transfected protein). The reversibility of the induced T250 phosphorylation is clear, with an observed half-life of approx. 5 minutes following a lag period. Thus, T250 phosphorylation is not an irreversible process, on the contrary turnover is relatively rapid. The detectability of endogenous PKCα has not proven a problem, with detection of TPA-induced PKCα phosphorylation by T(P)250 antiserum observed for ~4 x 10^4 cell equivalents. The actual use of this method for PKCα activation is evidenced by agonist treatment of Swiss 3T3 cells. Thus agents that can activate PKC (as judged for example by the induced phosphorylation and translocation of the PKC substrate MARCKS; see for example [Herget,

1994]) will induce the phosphorylation of PKCα at the T250 site. By contrast selective activation of the cAMP-dependent protein kinase does not.

One key advantage of this method as a detection device is that it should permit an assessment of activation in archival material where there is no opportunity to fractionate for a "translocation" assay. In fact as a routine measure of activation, following this PKCα autophosphorylation by Western (or a two-site ELISA) provides by far the most direct method for analysis. The ability to employ rapidly denatured samples without prior processing also bypasses any limitations imposed on the stability of membrane associated complexes. This limitation of a translocation assay may in part confound analysis of PKC responses to certain agonists such as insulin. However it is clearly demonstrated here that PKCα becomes phosphorylated at the T250 site in response to insulin.

As a general means of monitoring PKC activation, this site is conserved within the cPKC subclass and we have observed autophosphorylation of $PKC\beta_1$ and $PKC\beta_2$ on the equivalent sites employing suitable antisera. Thus, for the cPKC isotypes this appears to prove a general marker. The C2 domain is not conserved in the nPKC and aPKC subclasses although C2-like domains are present at the N-termini [Ponting, 1996]. Nevertheless both PKCδ and PKCε have been shown to become hyperphosphorylated in Swiss 3T3 cells following stimulation with mitogens [Olivier, 1994].

With respect to the T250 site itself and its location within the C2 domain, structure prediction [Srinivasan, 1996] places the T250 residue in a loop

adjacent to the four aspartic acid residues likely to be involved in co-ordination of Ca^{2+}. It is possible that phosphorylation at T250 alters the affinity for Ca^{2+} or perhaps modifies the manner in which Ca^{2+} can induce lipid interaction through this domain.

In conclusion, an *in vivo* and *in vitro* autophosphorylation site has been identified in PKCα. Antisera specific to the occupied site provide a facile assay for monitoring PKCα activation and this has been confirmed in a well characterised cell line. This analysis has been employed to demonstrate control of PKCα by insulin, a response that has proven difficult to monitor by other means. As an analytical method this approach will prove powerful in following PKC activation in archival samples and *in situ*.

Example 2: Identification of autophosphorylation site markers and reagents

The identification of autophosphorylation site markers for nPKC and aPKC isotypes involves the use of isolated recombinant enzymes (for example expressed in bacteria, insect cells or mammalian cells). These purified proteins are incubated under the conditions required for activation of each PKC, employing [γ^{32}P]-labelled ATP as substrate. Radioactivity incorporated into the kinase (autophosphorylation) is detected following SDS-PAGE separation and the labelled protein digested from the gel (or if desired following transfer to a membrane such as PVDF) with protease (trypsin is most efficient in this respect). Peptides derived from the digest are separated by HPLC and phosphorylation sites identified by a

combination of mass spectrometry, Edman degradation and phosphoaminoacid analysis.

Antibodies that specifically recognise the defined phosphorylation sites are obtained through immunisation (of for example rabbits or mice) with synthetic phospho-peptides (usually employing a seven amino acid peptide with the phosphorylated residue in the fourth position) coupled to a carrier (usually keyhole limpet haemocyanin). The antibodies so derived can then be employed to monitor PKC activation status.

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CLAIMS

1. A method of determining whether a protein kinase C (PKC) has been activated, the method comprising determining whether or to what extent a phosphorylatable site on the said protein kinase C, which site is associated with activation of said protein kinase C, has been phosphorylated.

2. A method according to Claim 1 wherein the protein kinase C is any one of an "a" class, or "c" class, or "n" class protein kinase C.

3. A method according to Claim 1 or 2 wherein the protein kinase C is any one of PKCα, PKCβ_1, PKCβ_2, PKC$_\gamma$, PKC$_\delta$, PKC$_\varepsilon$, PKC$_\theta$, PKC$_\eta$, PKC$_\mu$, PKC$_\zeta$, PKC$_\iota$ or PKC$_\lambda$.

4. A method according to Claim 3 wherein the protein kinase C is any one of PKCα, PKC$_{\beta 1}$, PKC$_{\beta 2}$ or PKC$_\gamma$.

5. A method according to Claim 3 wherein the phosphorylatable site is, or is equivalent to, threonine 250 (T250) of human PKC$_\alpha$.

6. A method according to any one of the preceding claims wherein the determination of whether or to what extent said phosphorylatable site has been phosphorylated is by contacting the protein kinase C with a reagent which is capable of distinguishing between the presence or absence of a phosphate moiety at said phosphorylatable site.

7. A method according to Claim 6 wherein the reagent is an antibody or a suitable fragment or derivative thereof which is capable of

distinguishing between the presence or absence of a phosphate within a particular amino acid sequence.

8. A method according to any one of the preceding claims further comprising distinguishing the said protein kinase C whose activation is to be determined from another protein kinase C.

9. A method according to Claim 8 wherein the protein kinase C whose activation is to be determined is separated from other protein kinases C before determining the phosphorylation state of the phosphorylatable site.

10. A reagent which is capable of distinguishing between the presence or absence of a phosphate moiety at a phosphorylatable site in a protein kinase C, which site is associated with activation of said protein kinase C.

11. A reagent according to Claim 10 which is an antibody or a suitable fragment or derivative thereof.

12. A reagent according to Claim 10 or 11 wherein the phosphorylatable site is, or is equivalent to, threonine 250 (T250) of bovine PKC_{α}.

13. Use of a reagent according to any one of Claims 10 to 12 for determining whether a protein kinase C has been activated.

14. A method for identifying a marker associated with activation of a protein kinase C the method comprising comparing the phosphorylation state of a phosphorylatable site in activated said protein kinase C with that of the phosphophorylatable site in non-activated said protein kinase and

determining those phosphorylatable sites whose phosphorylation state varies with the activation status of the protein kinase C.

15. A method according to Claim 15 wherein the phosphorylatable site is an autophosphorylatable site in the protein kinase C.

16. A kit of parts comprising a reagent which is capable of distinguishing between the presence or absence of a phosphate moiety at a phosphorylatable site in a protein kinase C, which site is associated with activation of said protein kinase C and, as controls, a sample comprising activated said protein kinase C and a sample comprising non-activated said protein kinase C.

17. A kit of parts according to Claim 16 further comprising a reagent capable of distinguishing the said PKC whose activation state is to be determined from another PKC.

18. A kit of parts according to Claim 16 or 17 wherein one or both of said reagents capable of distinguishing are antibodies.

19. A peptide comprising a phosphorylatable site in a protein kinase C which site is associated with activation of said protein kinase C, or a peptide in which said phosphorylatable site is phosphorylated.

20. A peptide according to Claim 19 containing between 5 and 50 amino acids.

21. A peptide according to Claim 20 containing between 8 and 20 amino acids.

22. A peptide according to any one of Claims 19 to 21 wherein the phosphorylatable site is, or is equivalent to, threonine 250 (T250) of PKC_{α}.

23. A peptide according to any one of Claims 19 to 22 consisting of the amino acids WDRTTRND.

24. A peptide according to any one of Claims 19 to 22 consisting of the amino acids WDRT(P)TRND.

25. An immunogenic formulation comprising a peptide according to any one of Claims 19 to 24 and a suitable carrier or adjuvant.

26. Any novel method of determining whether a protein kinase C has been activated as herein disclosed.

27. Any novel reagent which is capable of distinguishing between the presence or absence of a phosphate moiety at a phosphorylatable site in a protein kinase C, which site is associated with activation of said protein kinase C as herein disclosed.

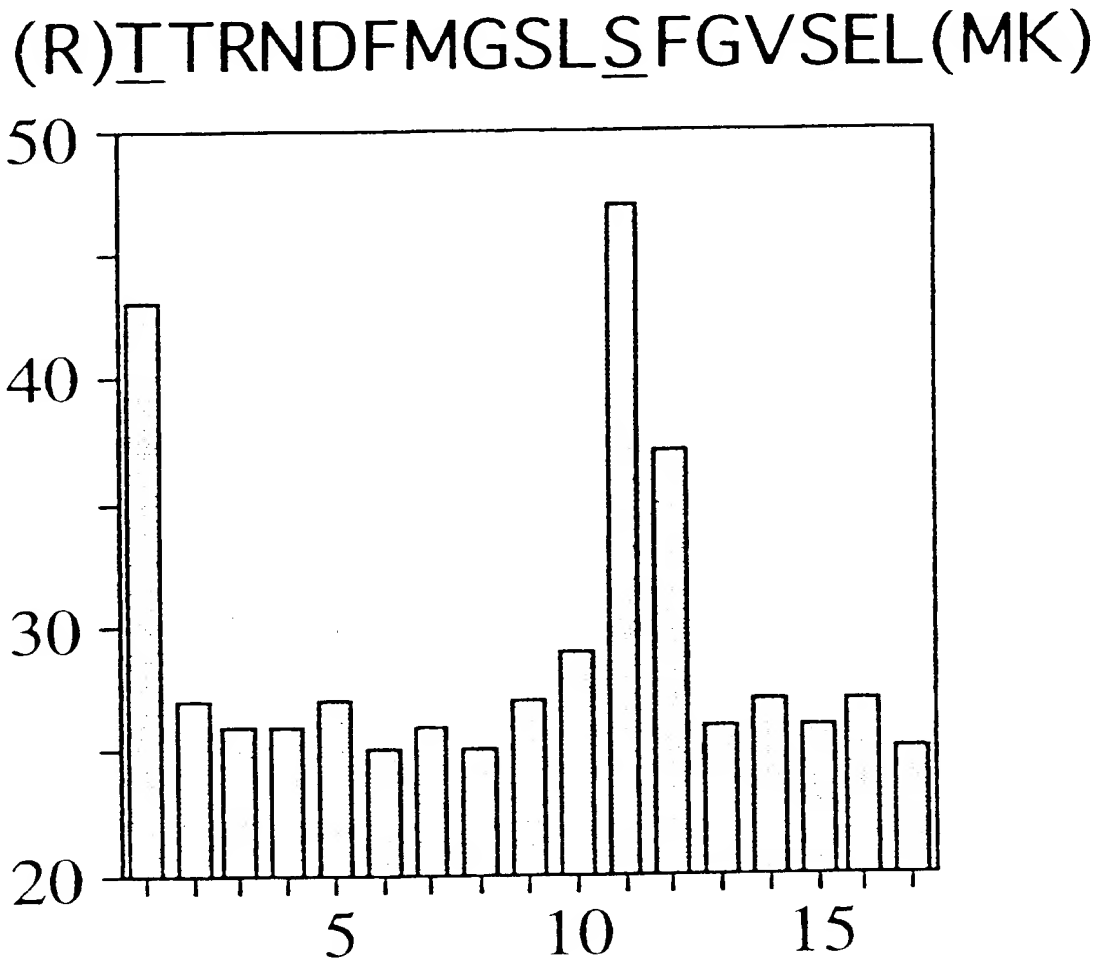


Fig. 1

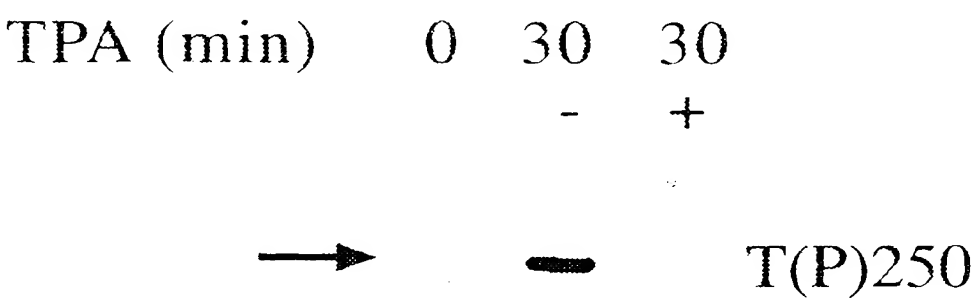
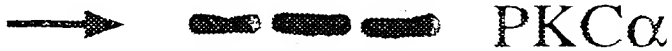


Fig. 2



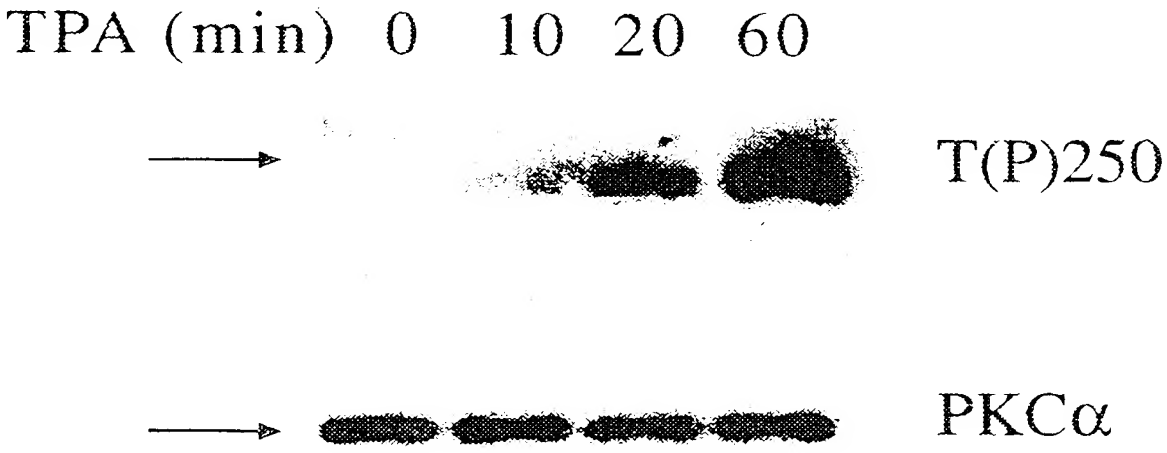
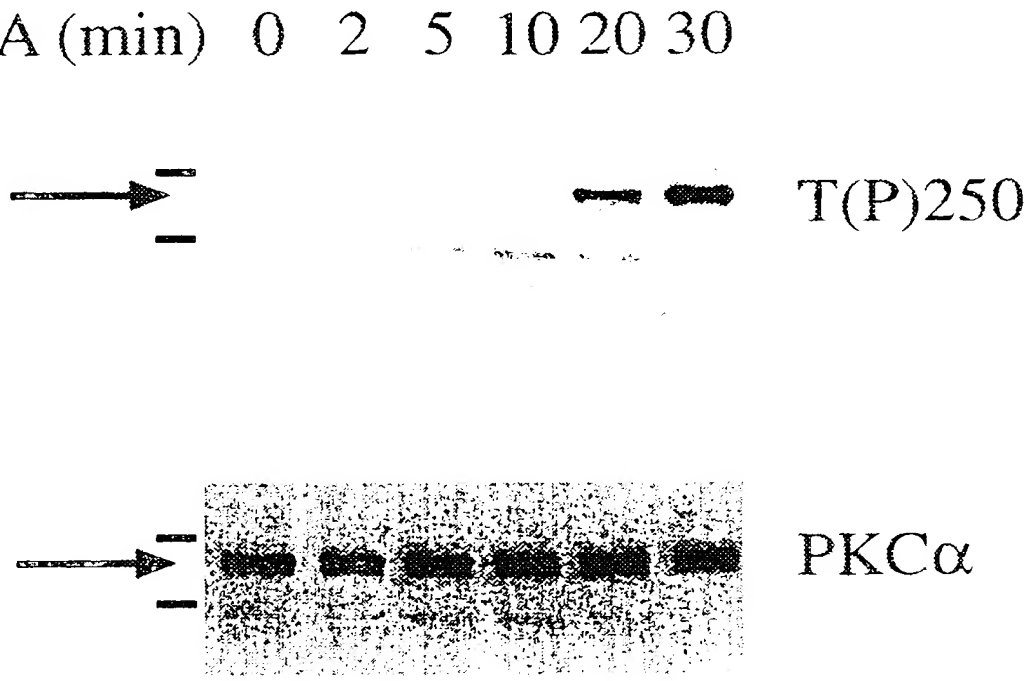


Fig. 3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T(P)250
PKCα

Fig. 4A

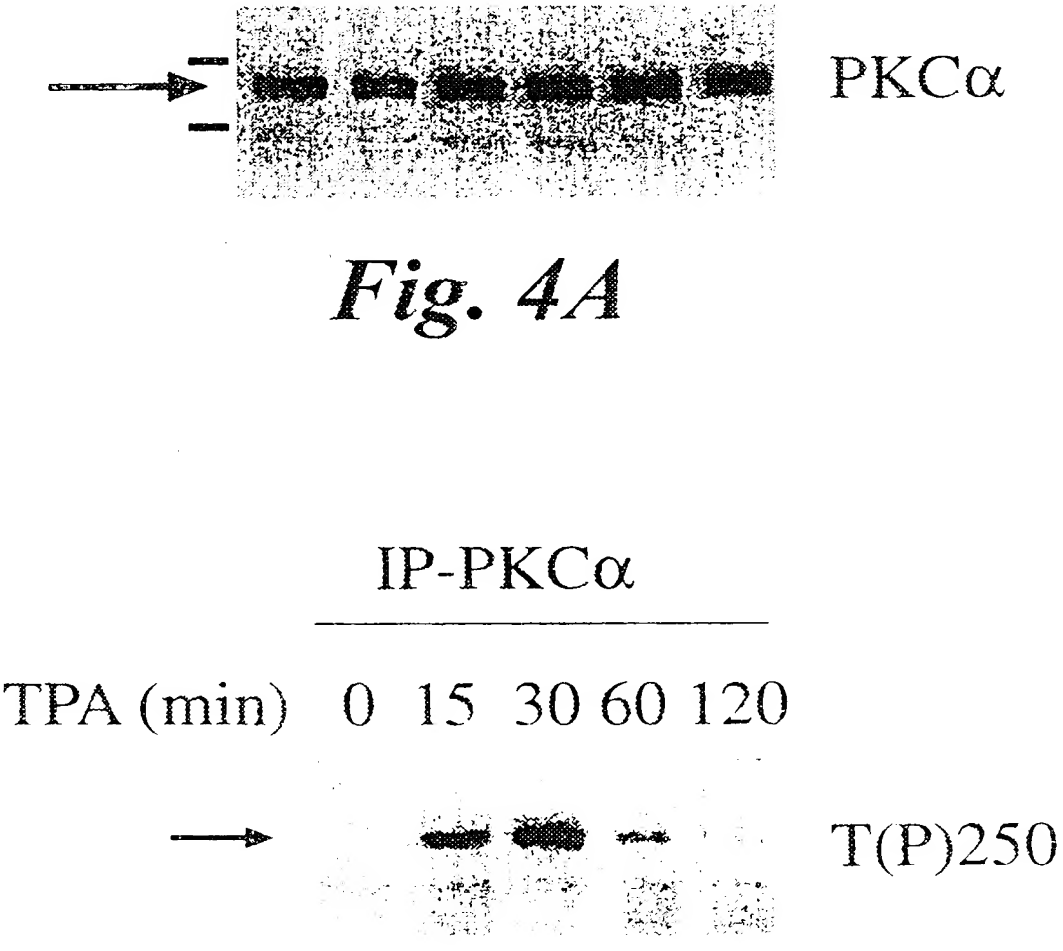

IP-PKCα
TPA (min) 0 15 30 60 120
T(P)250

Fig. 4B

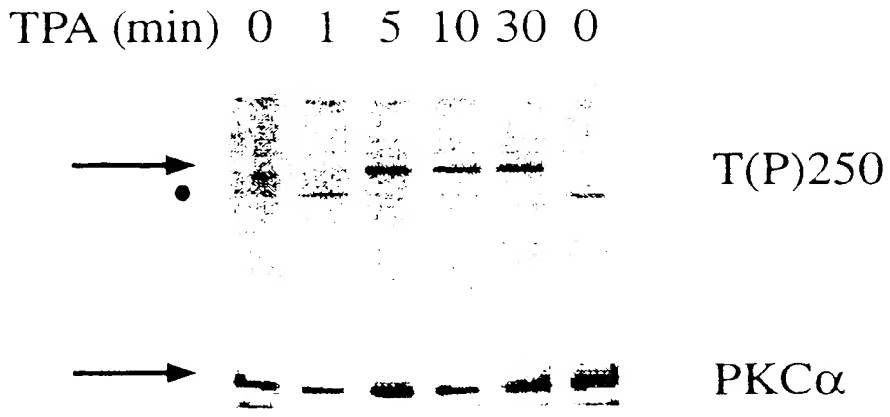

TPA (min) 0 1 5 10 30 0
T(P)250
PKCα

Fig. 5

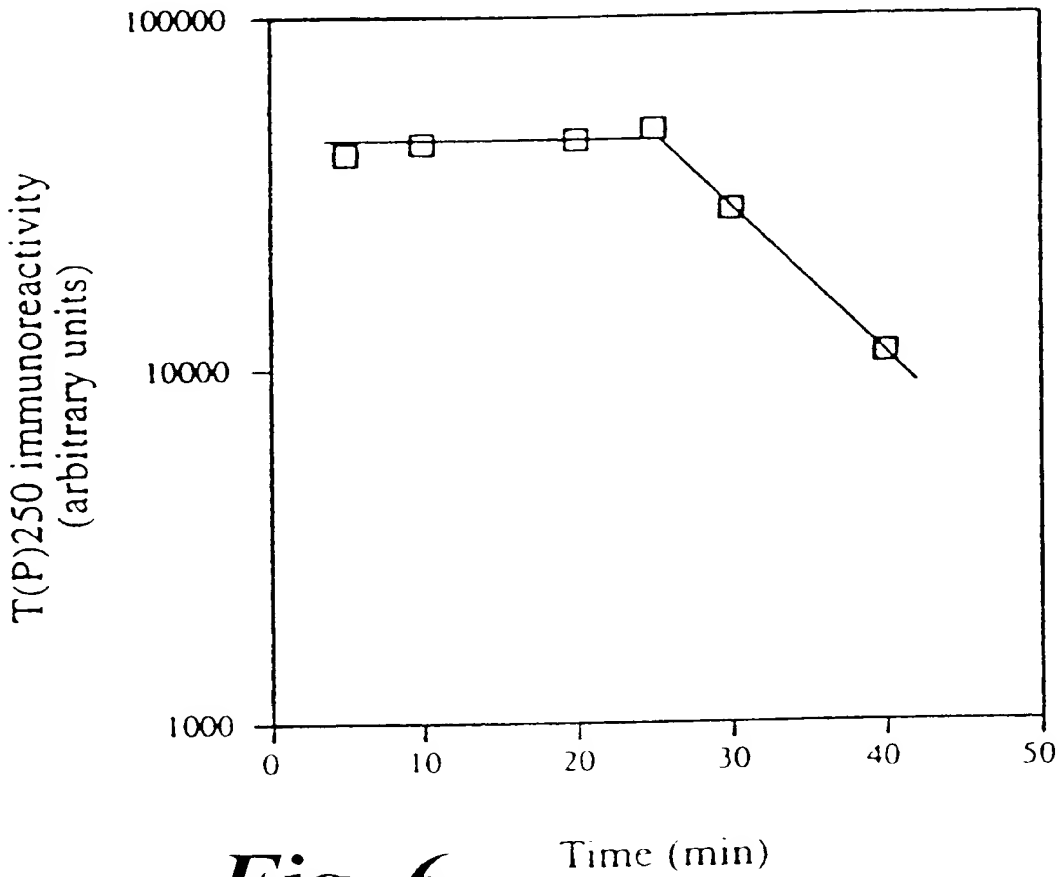
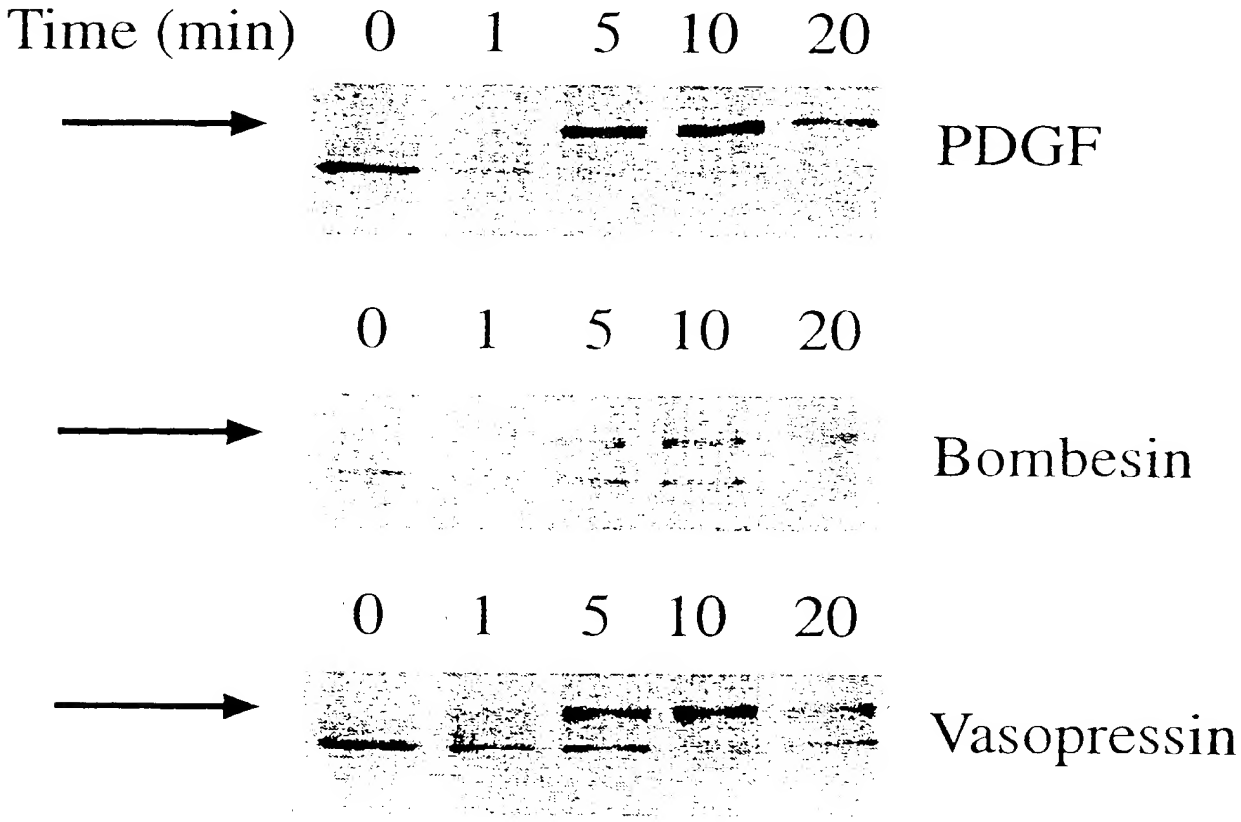

100000
10000
1000
T(P)250 immunoreactivity (arbitrary units)
0
10
20
30
40
50
Time (min)

Fig. 6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PDGF
0 1 5 10 20
Bombesin
0 1 5 10 20
Vasopressin

Fig. 7

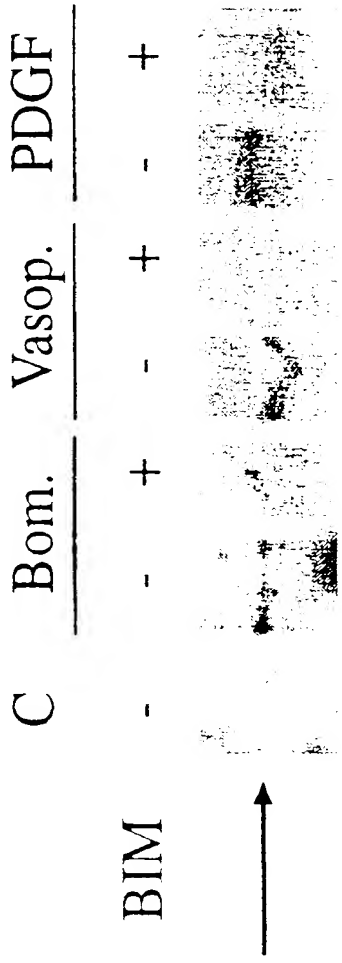
C
Bom.
Vasop.
PDGF
BIM
- - + - - +

Fig. 8

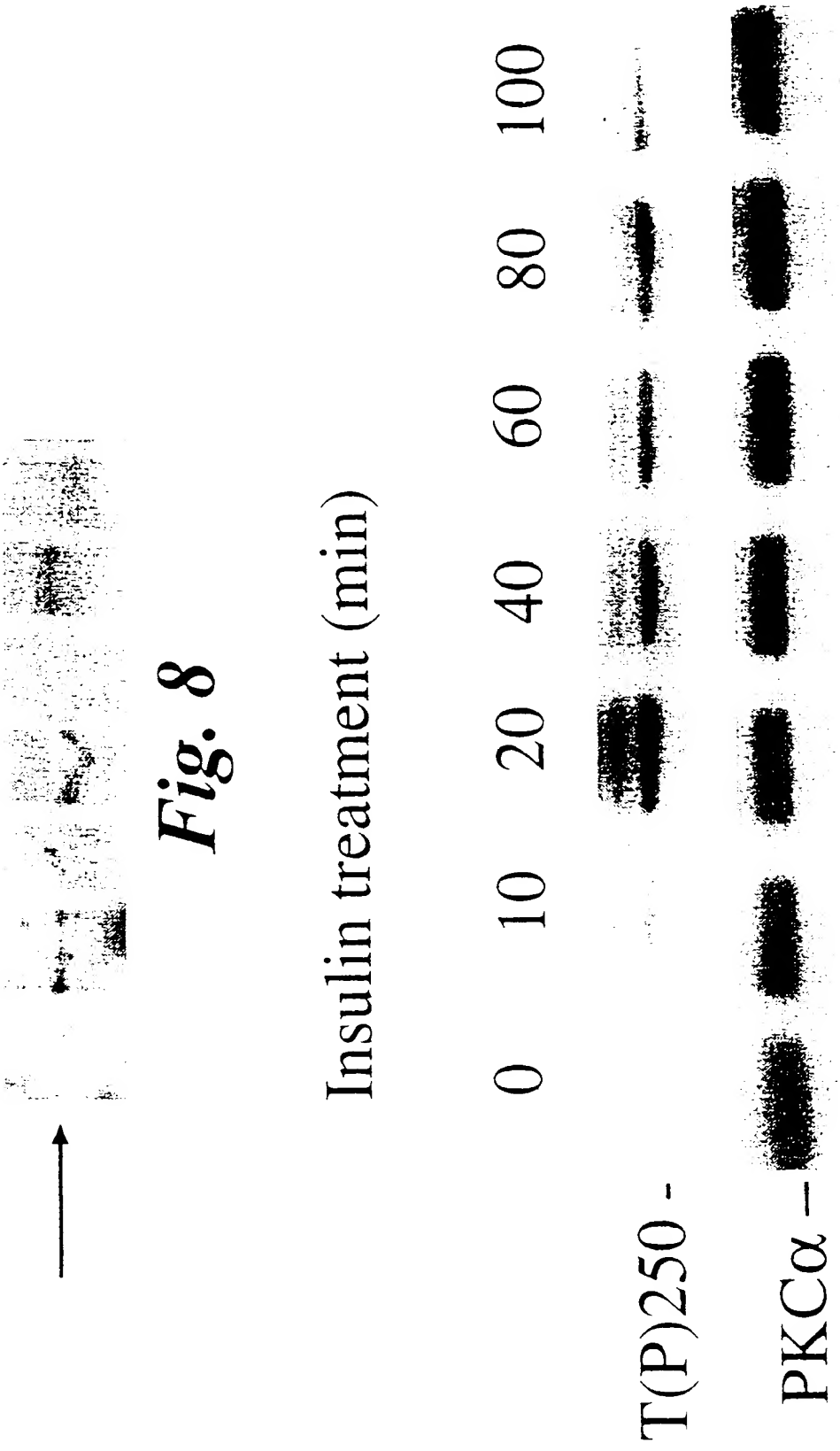
Insulin treatment (min)
0 10 20 40 60 80 100
T(P)250 -
PKCα –

Fig. 9

```
PKCALPHA(HUMAN)     MADVFPGNDSTASQDVANRFARKGALRQKNVHEVKDHKFIARFFKQPTFC
PKCBETA2(HUMAN)     MADPAAGPPPSEGEESTVRFARKGALRQKNVHEVKNHKFTARFFKQPTFC
PKCBETA1(HUMAN)     MADPAAGPPPSEGEESTVRFARKGALRQKNVHEVKNHKFTARFFKQPTFC
PKCGAMMA(RAT)       MAGLGPGGGDSEGGP-RPLFCRKGALRQKVVHEVKSHKFTARFFKQPTFC
                    ** .  .*  : .      *.******** ***** .*** **********

PKCALPHA(HUMAN)     SHCTDFIWGFGKQGFQCQVCCFVVHKRCHEFVTFSCPGADKGPDTDDPRS
PKCBETA2(HUMAN)     SHCTDFIWGFGKQGFQCQVCCFVVHKRCHEFVTFSCPGADKGPASDDPRS
PKCBETA1(HUMAN)     SHCTDFIWGFGKQGFQCQVCCFVVHKRCHEFVTFSCPGADKGPASDDPRS
PKCGAMMA(RAT)       SHCTDFIWGIGKQGLQCQVCSFVVHRRCHEFVTFECPGAGKGPQTDDPRN
                    *********:****:***** ****:********.****.*** :****.

PKCALPHA(HUMAN)     KHKFKIHTYGSPTFCDHCGSLLYGLIHQGMKCDTCDMNVHKQCVINVPSL
PKCBETA2(HUMAN)     KHKFKIHTYSSPTFCDHCGSLLYGLIHQGMKCDTCMMNVHKRCVMNVPSL
PKCBETA1(HUMAN)     KHKFKIHTYSSPTFCDHCGSLLYGLIHQGMKCDTCMMNVHKRCVMNVPSL
PKCGAMMA(RAT)       KHKFRLHSYSSPTFCDHCGSLLYGLVHQGMKCSCCEMNVHRRCVRSVPSL
                    ****::*:*.**************:******. * ****::** .****

PKCALPHA(HUMAN)     CGMDHTEKRGRIYLKAEVAD-EKLHVTVRDAKNLIPMDPNGLSDPYVKLK
PKCBETA2(HUMAN)     CGTDHTERRGRIYIQAHIDR-DVLIVLVRDAKNLVPMDPNGLSDPYVKLK
PKCBETA1(HUMAN)     CGTDHTERRGRIYIQAHIDR-DVLIVLVRDAKNLVPMDPNGLSDPYVKLK
PKCGAMMA(RAT)       CGVDHTERRGRLQLEIRAPTSDEIHITVGEARNLIPMDPNGLSDPYVKLK
                    ** ****:***: :: .    : : : * :*:**:***************

PKCALPHA(HUMAN)     LIPDPKNESKQKTKTIRSTLNPQWNESFTFKLKPSDKDRRLSVEIWDWDR
PKCBETA2(HUMAN)     LIPDPKSESKQKTKTIKCSLNPEWNETFRFQLKESDKDRRLSVEIWDWDL
PKCBETA1(HUMAN)     LIPDPKSESKQKTKTIKCSLNPEWNETFRFQLKESDKDRRLSVEIWDWDL
PKCGAMMA(RAT)       LIPDPRNLTKQKTKTVKATLNPVWNETFVFNLKPGDVERRLSVEVWDWDR
                    *****:. :******:::***  ***:* *:**  .* :******:****
                250 ∤(α)
PKCALPHA(HUMAN)     TTRNDFMGSLSFGVSELMKMPASGWYKLLNQEEGEYYNVPIP-EGDEE--
PKCBETA2(HUMAN)     TSRNDFMGSLSFGISELQKAGVDGWFKLLSQEEGEYFNVPVPPEGSE---
PKCBETA1(HUMAN)     TSRNDFMGSLSFGISELQKASVDGWFKLLSQEEGEYFNVPVPPEGSE---
PKCGAMMA(RAT)       TSRNDFMGAMSFGVSELLKAPVDGWYKLLNQEEGEYYNVPVA-DADNCSL
                    *:******::***:***  *  ..**:***.******:***:. :.:

PKCALPHA(HUMAN)     -----GNMELRQKFEKAKLGPAGNKVISP---SEDRK-QPSN-NLDRVKL
PKCBETA2(HUMAN)     -----ANEELRQKFERAKIS-QGTKVPEEKTTNTVSK-FDNNGNRDRMKL
PKCBETA1(HUMAN)     -----ANEELRQKFERAKIS-QGTKVPEEKTTNTVSK-FDNNGNRDRMKL
PKCGAMMA(RAT)       LQKFEACNYPLELYERVRMGPSSSPIPSPSPSPTDSKRCFFGASPGRLHI
                          .    : :*:.:::. .. : .          *    . . .*:::

PKCALPHA(HUMAN)     TDFNFLMVLGKGSFGKVMLADRKGTEELYAIKILKKDVVIQDDDVECTMV
PKCBETA2(HUMAN)     TDFNFLMVLGKGSFGKVMLSERKGTDELYAVKILKKDVVIQDDDVECTMV
PKCBETA1(HUMAN)     TDFNFLMVLGKGSFGKVMLSERKGTDELYAVKILKKDVVIQDDDVECTMV
PKCGAMMA(RAT)       SDFSFLMVLGKGSFGKVMLAERRGSDELYAIKILKKDVIVQDDDVDCTLV
                    :**.**************:::*:*::****:*******::*****:**:*

PKCALPHA(HUMAN)     EKRVLALLDKPP-----FLTQLHSCFQTVDRLYFVMEYVNGGDLMYHIQQ
PKCBETA2(HUMAN)     EKRVLALPGKPP-----FLTQLHSCFQTMDRLYFVMEYVNGGDLMYHIQQ
PKCBETA1(HUMAN)     EKRVLALPGKPP-----FLTQLHSCFQTMDRLYFVMEYVNGGDLMYHIQQ
PKCGAMMA(RAT)       EKRVLALGGRGPGSRPHFLTQLHSTFQTPDRLYFVMEYVTGGDLMYHIQQ
                    *******  .: *     ******* *** ********** **********

PKCALPHA(HUMAN)     VGKFKEPQAVFYAAEISIGLFFLHKRGIIYRDLKLDNVMLDSEGHIKIAD
PKCBETA2(HUMAN)     VGRFKEPHAVFYAAEIAIGLFFLQSKGIIYRDLKLDNVMLDSEGHIKIAD
PKCBETA1(HUMAN)     VGRFKEPHAVFYAAEIAIGLFFLQSKGIIYRDLKLDNVMLDSEGHIKIAD
PKCGAMMA(RAT)       LGKFKEPHAAFYAAEIAIGLFFLHNQGIIYRDLKLDNVMLDAEGHIKITD
                    :******:*.**********:.**********:********:*********
```

Fig. 10 (Part 1)

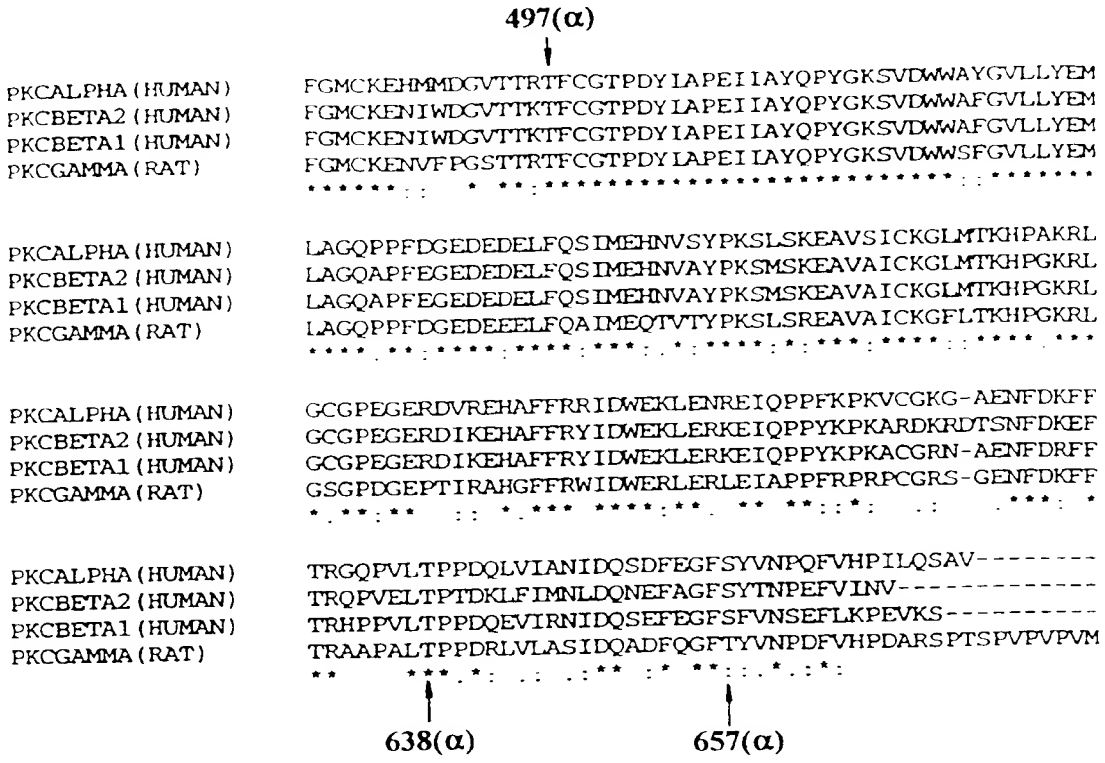
497(α)
PKCALPHA(HUMAN) FGMCKEHMMDGVTTRTFCGTPDYIAPEIIAYQPYGKSVDWWAYGVLLYEM
PKCBETA2(HUMAN) FGMCKENIWDGVTTKTFCGTPDYIAPEIIAYQPYGKSVDWWAFGVLLYEM
PKCBETA1(HUMAN) FGMCKENIWDGVTTKTFCGTPDYIAPEIIAYQPYGKSVDWWAFGVLLYEM
PKCGAMMA(RAT) FGMCKENVFPGSTTRTFCGTPDYIAPEIIAYQPYGKSVDWWSFGVLLYEM
PKCALPHA(HUMAN) LAGQPPFDGEDEDELFQSIMEHNVSYPKSLSKEAVSICKGLMTKHPAKRL
PKCBETA2(HUMAN) LAGQAPFEGEDEDELFQSIMEHNVAYPKSMSKEAVAICKGLMTKHPGKRL
PKCBETA1(HUMAN) LAGQAPFEGEDEDELFQSIMEHNVAYPKSMSKEAVAICKGLMTKHPGKRL
PKCGAMMA(RAT) LAGQPPFDGEDEEELFQAIMEQTVTYPKSLSREAVAICKGFLTKHPGKRL
PKCALPHA(HUMAN) GCGPEGERDVREHAFFRRIDWEKLENREIQPPFKPKVCGKG-AENFDKFF
PKCBETA2(HUMAN) GCGPEGERDIKEHAFFRYIDWEKLERKEIQPPYKPKARDKRDTSNFDKEF
PKCBETA1(HUMAN) GCGPEGERDIKEHAFFRYIDWEKLERKEIQPPYKPKACGRN-AENFDRFF
PKCGAMMA(RAT) GSGPDGEPTIRAHGFFRWIDWERLERLEIAPPFRPRPCGRS-GENFDKFF
PKCALPHA(HUMAN) TRGQPVLTPPDQLVIANIDQSDFEGFSYVNPQFVHPILQSAV--------
PKCBETA2(HUMAN) TRQPVELTPTDKLFIMNLDQNEFAGFSYTNPEFVINV----------
PKCBETA1(HUMAN) TRHPPVLTPPDQEVIRNIDQSEFEGFSFVNSEFLKPEVKS----------
PKCGAMMA(RAT) TRAAPALTPPDRLVLASIDQADFQGFTYVNPDFVHPDARSPTSPVPVPVM
638(α)
657(α)

Fig. 10 (Part 2)

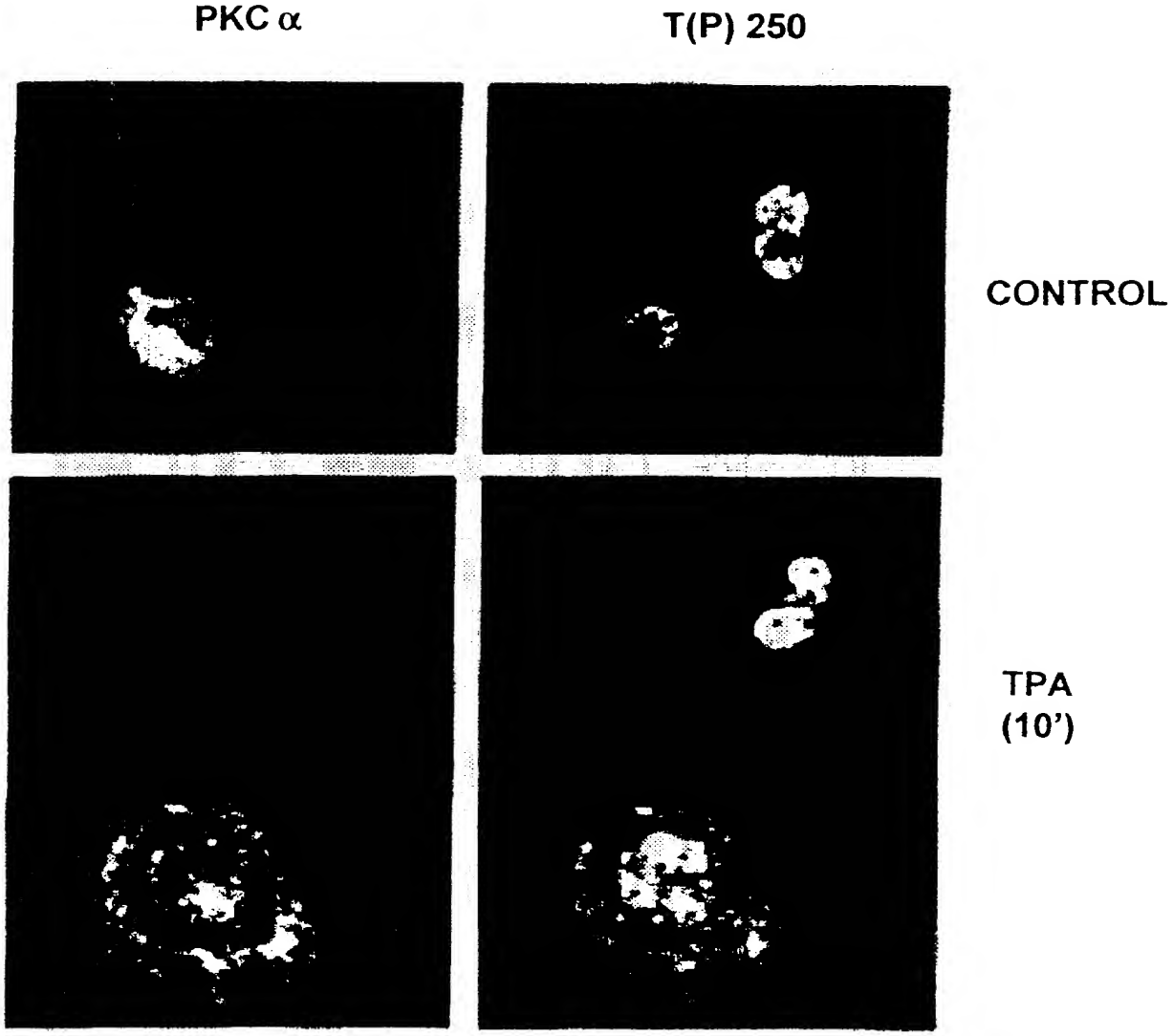

Fig. 11

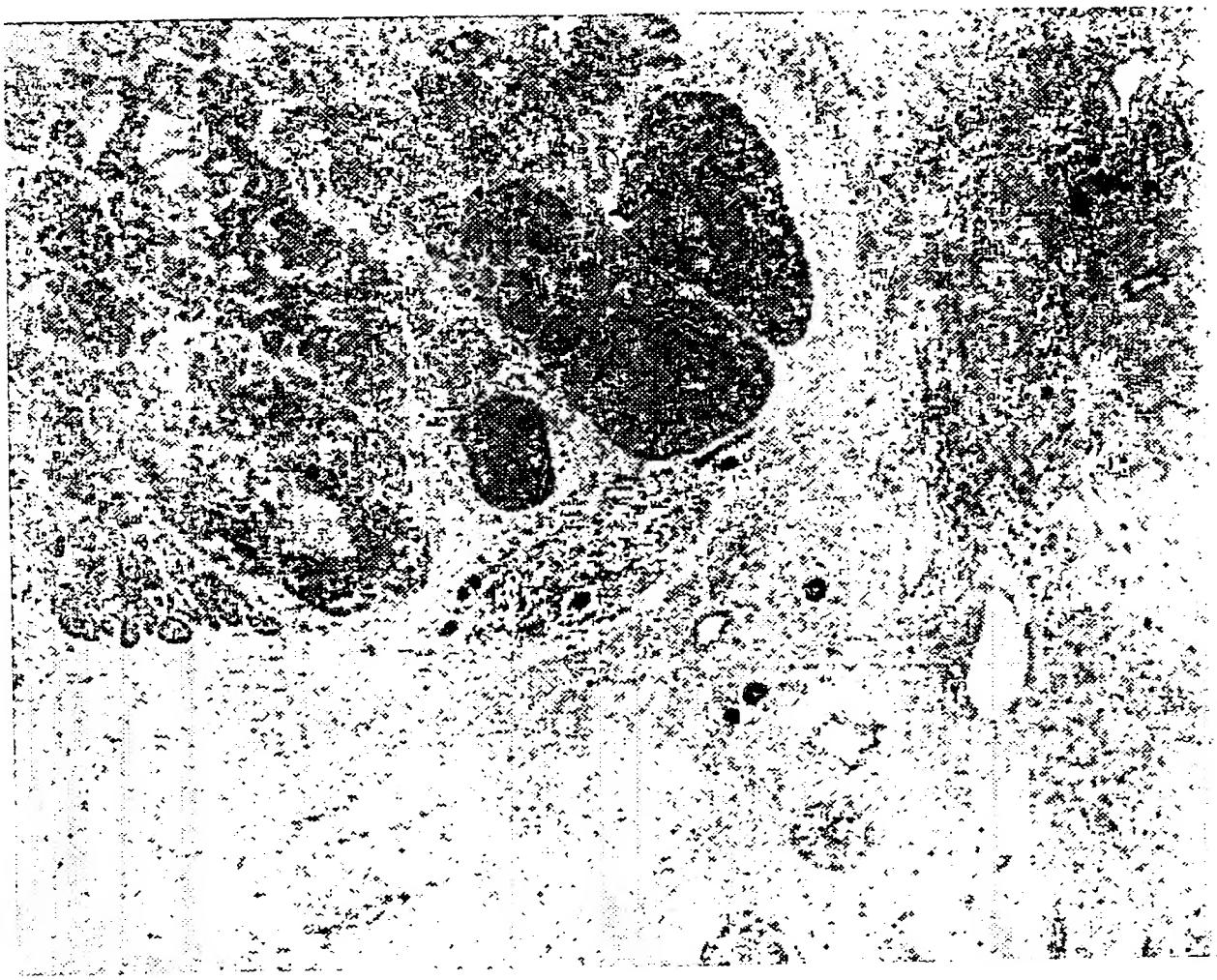

Fig. 12

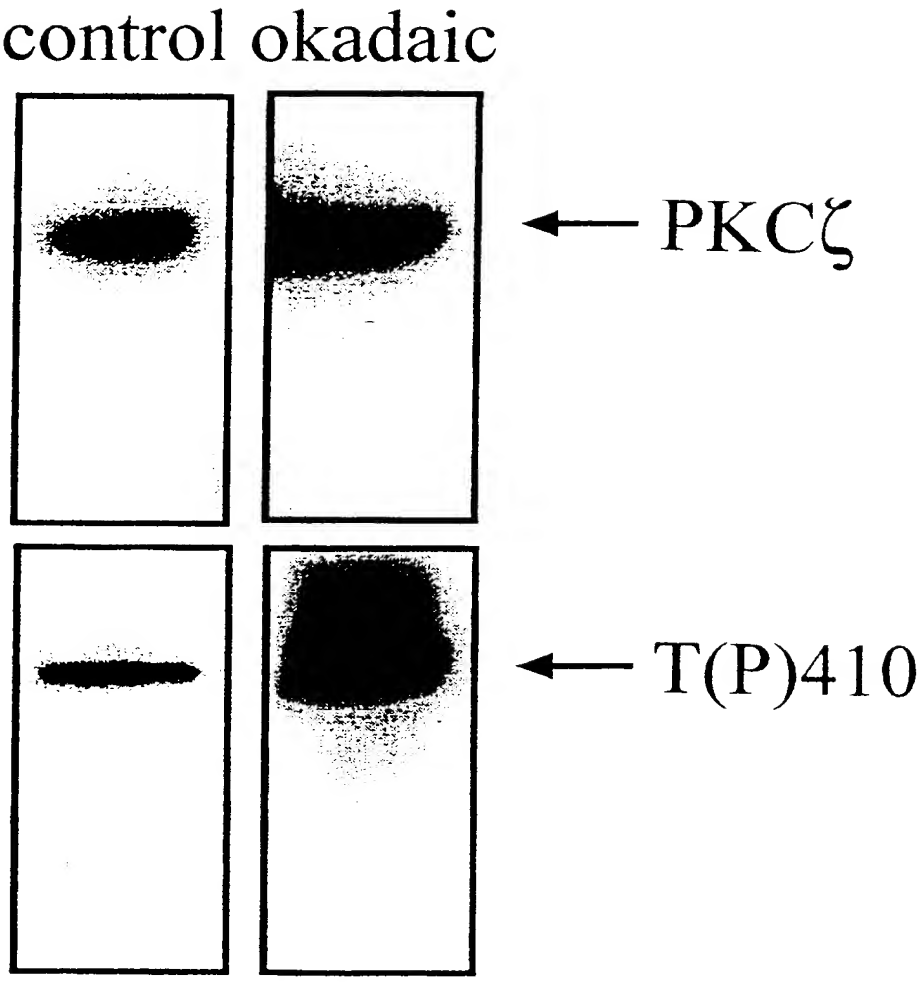

Fig. 13

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/00510

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/573 C12N9/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	TSUTAKAWA, S. E.: "Determination of in Vivo Phosphorylation Sites in Protein Kinase C" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 45, 10 November 1995 (1995-11-10), pages 26807-26812, XP002109455 cited in the application	1-4, 19-22, 26,27
A	abstract page 26808, column 1, paragraph 6; figures 1,6	5,6,23, 24

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[X] Further documents are listed in the continuation of box C

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 July 1999	03/08/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gundlach, B

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INTERNATIONAL SEARCH REPORT

Interi. ..onal Application No
PCT/GB 99/00510

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
X	BORNANCIN, F. ET AL.: "Phosphorylation of threonine 638 critically controls the dephosphorylation and inactivation of protein kinase C-alpha" CURRENT BIOLOGY, vol. 6, no. 9, 1 September 1996 (1996-09-01), pages 1114-1123, XP002109456 cited in the application abstract	1-4,8,9, 14,15
X	MITCHELL, F.E. ET AL.: "The phosphorylation of protein kinase C as a potential measure of activation" THE BIOCHEMICAL JOURNAL, vol. 261, no. 1, 1 July 1989 (1989-07-01), pages 131-136, XP002109457 cited in the application abstract	1-4,14, 15
X	KERANEN, L.M. ET AL.: "Protein kinase C is regulated in vivo by three functionally distinct phosphorylations" CURRENT BIOLOGY, vol. 5, no. 12, 1 October 1995 (1995-10-01), pages 1394-1403, XP002109458 cited in the application abstract	1-4,14, 15
X	CAZAUBON, S. ET AL.: "Threonine-497 is a critical site for permissive activation of protein kinase C-alpha" BIOCHEMICAL JOURNAL, vol. 301, no. 2, 15 July 1994 (1994-07-15), pages 443-448, XP002109459 cited in the application abstract	1-4
A	LINDBERG R A ET AL: "IDENTIFICATION OF cDNA CLONES THAT CODE FOR PROTEIN-TYROSINE KINASES BY SCREENING EXPRESSION LIBRARIES WITH ANTIBODIES AGAINST PHOSPHOTYROSINE" ONCOGENE, vol. 3, no. 6, 1 December 1988 (1988-12-01), pages 629-633, XP000196785 ISSN: 0950-9232 abstract	1,6

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INTERNATIONAL SEARCH REPORT

Inter. .onal Application No
PCT/GB 99/00510

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate of the relevant passages	Relevant to claim No
A	LINDBERG, R.A.: "Characterization of a human protein threonine kinase isolated by screening an expression library with antibodies to phosphotyrosine" ONCOGENE, vol. 8, no. 2, February 1993 (1993-02), pages 351-359, XP002109460 abstract	1,6
A	CAZAUBON, SYLVIE ET AL: "Effector-dependent conformational changes in protein kinase C-gamma through epitope mapping with inhibitory monoclona antibodies" EUR. J. BIOCHEM., 194(3), 799-804 , October 1990 (1990-10), XP002109461 cited in the application abstract	1,6

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